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13. ABSTRACT *(Maximum 200 words)*

Tubulin, the major protein of microtubules, the cytoskeletal structures that mediate cell division, is the target for the action of several anti-cancer drugs which are routinely used for cancer chemotherapy. Different isoforms of tubulin are expressed differently in tissues, which makes each tissue unique with respect to its composition of tubulin isoforms. Tubulin also undergoes various post-translational modifications *in vivo*. The purpose of this proposal is to characterize the tubulin isoforms and their post-translational modifications in breast cancer cell lines. These studies may identify cancer-specific modifications in tubulin isoforms which could be used as prognostic markers for the detection of breast cancer. We have prepared a monoclonal antibody that can recognize the major α–tubulin in the breast cancer cells. Results of immunoblotting with a monoclonal antibody specific for β_{II} tubulin isoform show that β_{II} tubulin, which is the major isoform in the brain, is absent in both MCF-7 and MDA-MB-231 cell lines. Preliminary studies with the antitumor drugs colchicine, podophyllotoxin, paclitaxel, vinblastine and maytansine show that maytansine is the most potent followed by paclitaxel and podophyllotoxin. Future studies will help identify breast cancer-specific drugs and possibly may lay the platform for designing drugs specific for breast cancer.

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A Banerjee 5/27/1999

PI - Signature Date

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Introduction

Microtubules, the cylindrical eukaryotic organelles, mediate cell division and a variety of cellular functions. Tubulin, the building-block protein of microtubules, is the target for the action of several anti-cancer drugs such as *Vinca* alkaloids, and paclitaxel which are potent antitumor agents and are routinely used for cancer chemotherapy. Tubulin, the 100 Kda subunit protein of microtubules exists in different tissues as several isoforms which differ significantly in their drug-binding properties. Different isoforms of tubulin are expressed differently in tissues, which makes each tissue unique with respect to its composition of tubulin isoforms. Tubulin also undergoes post-translational modifications *in vivo*. These include tyrosination-detyrosination and acetylation of α-tubulin; poly-glutamylation, in which up to six Glu residues are added in a pseudo peptide linkage near the C-terminal of α– and β–tubulin; poly-glycylation and phosphorylation of one isoform of β–tubulin. In this proposal, I will characterize the tubulin isoforms and their post-translational modifications in breast cancer cell lines. These studies may identify cancer-specific modifications in tubulin isoforms which could be used as prognostic markers for the detection of breast cancer. Various anti-cancer drugs such as *Vinca* alkaloids, different taxanes and thiocolchicine analogs will be tested for their inhibition of growth for those cell lines. The antitumor drugs will be tested for their interactions with different tubulin isoforms. These studies will help identify breast cancer-specific drugs and possibly may lay the platform for designing drugs specific for breast cancer.

Approved Statement of Work

TASK 1: MONTHS 1 - 6:

- To grow the breast cancer cells
- To isolate tubulin from the breast cancer cells by paclitaxel-induced assembly
- To quantitate each β-tubulin isoform by SDS-PAGE and immunoblotting

TASK 2: MONTHS 7 - 12:

- To grow the breast cancer cells
- To isolate tubulin from the breast cancer cells by paclitaxel-induced assembly
- To study the post-translational modifications of tubulin

TASK 3: MONTHS 13 - 20:

- To grow the breast cancer cells in the presence of antitumor drugs
- To determine the IC_{50} values for different antitumor drugs

TASK 4: MONTHS 21 - 36:

- To study the interaction of anti-tumor drugs with purified tubulin isoforms from bovine brain
- To study the drug effects on the assembly and dynamics of microtubules

Interaction of novel thiocolchicine analogs with the β-tubulin isoforms

This part of the report is in response to the Task 4. Since the experiments outlined in Tasks 1 and 2 of the approved Statement of Work are slow in nature (such as growing cancer cells in bulk quantities), I have initiated these experiments much earlier in order to finish before schedule.

The thiocolchicines are recently developed drugs which are found to possess differential anti-tumor properties to different solid tumors as well as tumor cell lines (1). The binding of the drugs to tubulin were measured by studying the intrinsic tryptophan fluorescence of tubulin. Both drugs quench the fluorescence of tubulin in a concentration dependent manner (2).

The affinity constants were measuerd by studying the fluorescence quenching at different drug concentrations. The results show that the binding of THC 5 to tubulin followed a simple one-affinity binding model with a K_d value of 6 μM. In contrast, the binding of THC 18 followed a two-affinity model. In this case, the binding data showed two affinities: one high-affinity with a K_d value of 0.025 μM and a low affinity with a K_d value of 17 μM. This means that THC 18 interacts differently with different tubulin isoforms present in brain tubulin; the affinity for one isoform seems to be 680-fold higher than the others. Thus this drug should be extremely potent for the cells which contain the faster binding tubulin isoform, while it will be inactive to those cells which contain primarily the slower binding isoforms. In future, other thiocolchicine analogs will also be studied.

The results of the studies involving the thiocolchicine analogs demonstrate that tubulin isoforms $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$ may differ in their interactions with some of these drugs. These results might also be important for the development of colchicine analogues specific for a particular tubulin isoform. Since some of the tubulin isoforms are predominant in certain tissues, a colchicine analogue specific for a tubulin isoform may be useful for tissue-specific chemotherapy.

This part of the report was presented as a poster in the 38th Annual Meeting of the American society for Cell Biology in san Francisco, CA, (December 12-16, 1998). The abstract is published in: Mol. Biol. Cell 9, p409a. Part of the results are published in:
Biochem. Biophys. Res. Commun. 254, 334-337

Characterization of the monoclonal antibody AYN.6D10 by immunoblotting:

A monoclonal antibody was generated by using a synthetic peptide corresponding to the COOH-terminal sequence of tyrosinated α1/2–tubulin. This is related to the experiments outlined in Task 4. The antibody was generated to isolate different α–tubulin isoforms. The antibody was tested by SDS-PAGE and Western blot using fusion proteins corresponding to the C-terminal sequences of different α-tubulin isoforms. The fusion proteins (kind gifts from Dr. Nicholas J. Cowan, New York University Medical Center) were made by fusing the cDNA sequence corresponding to the C-terminal portion of each α-tubulin (amino acid 254 to the C-terminal end for Mα1/2, Mα3/7, and Mα6 and amino acid 168 to the C-terminal end for Mα4 and Mα4+Y) with the *E. coli* trp E gene. The C-terminal Tyr residue was added to Mα4 by site-directed mutagenesis. The fusion proteins were expressed in *E. coli* and were purified from the bacterial extract. As shown in the Fig 6, the antibody recognized Mα1/2 , Mα3/7, Mα6 and the tyrosinated form of Mα4. The only isoforms those were not recognized by the antibody is the non-tyrosinated form of Mα4 and the detyrosinated form of Mα3/7.

Chromatography of bovine brain tubulin on the antibody-Sepharose column: PC-tubulin was passed through the immunoaffinity column and the unbound fraction was pooled. This unbound fraction will be refered to as the fraction A. After collection of the unbound fraction, the column was extensively washed with the buffer and the bound fraction was eluted with a linear salt gradient of 0-1 M NaCl. The bound protein eluted in two peaks, I and II. Fractions from the peaks I and II will be referred to as fraction B and fraction C respectively and were pooled separately. The column was then washed with 1M NaCl and the remaining bound protein was eluted with 3M KI. The KI-eluted fraction will be referred to as "fraction D ". All the fractions were subjected to SDS-PAGE and immunoblotting after reduction and carboxymethylation. The immunoblot results show that the α-tubulin band from the unbound fracion was not recognized by the monoclonal antibody AYN.6D10. Furthermore, the NaCl-eluted peak II and the KI-eluted fraction were recognized by the antibody. The NaCl-eluted peak I exhibited very weak signal which might be due to the overlapping of peaks I and II. Studies are under way to identify the above fractions. Subsequently, assembly and the drug-binding studies will be performed.

Purification of α-tubulin isoforms by using an immunoaffinity column:

By using the antibody an immunoaffinity column was prepared. When bovine brain tubulin was chromatographed on this column, three α-tubulin isoforms A, B, and C were purified in functionally active state. The α–tubulin from these isoforms were identified by sequence analysis using automated Edmann degradation method. 'A' was found to contain the detyrosinated form of Mα1/2; 'B' contains a mixture of the detyrosinated forms of Mα1/2 and Mα4; 'C' contains the tyrosinated form of Mα1/2.

Differential interaction of taxol with the α-tubulin isoforms:

Paclitaxel (PTX) is known to induce the assembly of PC-tubulin in the absence of microtubule-associated proteins. In an effort to study whether the α–tubulin is involved in the drug interaction, we have studied the *in vitro* assembly of the isoforms in the presence of PTX. It is evident from the figure (Fig 2) that different α-tubulin isoforms exhibit different assembly kinetics. As shown in the figure, the lowest rate and extent of assembly was observed for the unfractionated tubulin and inceased in the following order: tyrosinated Mα1/2 >nontyrosinated (Mα1/2 + Mα4) >tyrosinated Mα1/2. The initial rates of assembly are 0.026, 0.039, 0.124, and 0.124 A_{350} units per minute for PC-tubulin, fraction A, fraction B, and fraction C respectively. Thus, for the tyrosinated Mα1/2 (fraction C), the initial rate of assembly is almost 4.8-fold higher than that of the PC-tubulin. The extent of assembly for tyrosinated Mα1/2 is twice as much as that of the unfractionated tubulin. Electron microscopic examination of the negatively stained samples confirms the formation of polymers resembling normal microtubules. Thus, we find that the isotypically pure α-tubulin isoforms differ significantly in their assembly kinetics in the presence of paclitaxel. The tyrosinated Mα1/2 isoform (fraction C) assembled much better than the nontyrosinated Mα1/2 (fraction A), while a mixture of nontyrosinated Mα1/2 and Mα4 isoforms assembled at intermediate rates.

Since the fraction C apparently differs from fraction A only in the C-terminal tyrosine, it is difficult to understand how such a small difference may affect the PTX binding. One possible explanation may be that the isoforms may differ in their post-translational modifications. It is known that α–tubulin undergoes post-translational modifications such as acetylation, polyglutamylation, and polyglycylation. The acetylation is known to occur at the Lys^{40}, while the polyglutamylation occurs on Glu^{445} of α–tubulin. We have checked the acetylation and polyglutamylation pattern of the α-tubulin isoforms. We find that the fractions B and C are twice as acetylated as fraction A (data not shown). Our preliminary mass spectrometric studies revealed that the tyrosinated Mα1/2 (fraction C) is post-translationally modified with side chains of 1-4 Glu residues through the γ–carboxyl of Glu^{445}. In contrast, the

nontyrosinated Mα1/2 (fraction A) contains 1-3 Gly residues (unpublihsed data). These results indicate that the PTX-binding domain on the α–tubulin may be in close proximity with either the site of acetylation (Lys^{40}) or that of polyglutamylation (Glu^{445}). Although at present it is not clear what makes the isoforms so different with respect to PTX interaction, nonetheless, our results clearly indicate that a part of the PTX-binding domain may be on α–tubulin.

This part of the report was presented as two posters in the 38th Annual Meeting of the American society for Cell Biology in san Francisco, CA, (December 12-16, 1998). The abstracts are published in:

1. **Mol. Biol. Cell 9, p149a. Ab#864**
2. **Mol. Biol. Cell 9, p150a. Ab#869**

The complete work was published in:
Biochemistry , 38, 5438-5446, April 27, 1999.

Purification of tubulin from MCF-7 and MDA-MB-231 cell lines:

In response to Tasks 1 & 2, breast cancer cells MCF-7 and MDA-MB-231 cell lines were grown in 150 ml culture flasks in media containing 10% fetal bovine serum. After the flasks became confluent, the medium was discarded and the cells were washed with cold PBS buffer. The cells were then trypsinized, washed twice with PBS buffer and were harvested. The cells were stored as pellets in liquid nitrogen.

About 1 ml packed volume of Cells was mixed with equal volume of buffer A [0.1 M Mes-Na (pH 6.4), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM GTP , 2 mM PMSF and a coctail of protease inhibitors], and was homogenized using a glass-teflon motorized homogenizer. The homogenate was centrifuged at 4^0C at 100,000 g for 1 h. The cell extract (supernatant) was carefully aspirated without disturbing the pellet, and was incubated in the presence of 10 μM paclitaxel at 37^0C for 30 min to form microtubules. The microtubules were harvested by centrifuging the mixture at 100,000 g for 1 h at 25^0C. Tiny amounts of microtubule pellets were obtained from both the MCF-7 and MDA-MB-231 cells.

The microtubule pellets were resuspended in buffer, boiled with Laemmli sample buffer and were subjected to SDS-polyacrylamide gel electrophoresis. An identical gel was transferred onto nitrocellulose membrane and was processed for immunoblotting with different antibodies specific for α– and β–tubulin. The results of immunoblotting with anti-β_{II} antibody (Fig 1) show that tubulin from either MCF-7 or MDA-MB-231 cells contains absolutely no β_{II} isoform of tubulin, which is the major β–tubulin isoform in brain. The results of immunoblotting with anti-tyrosinated tubulin antibody show that tubulin from the breast cancer cells contain much higher amounts of tyrosinated tubulin than that of the brain tubulin. Although these data are a bit preliminary, they may indicate that the tyrosination of α–tubulin, a well-known post-translational modification, may occur much more in the breast cancer cells. A detailed study employing normal MCF-10F cells will be performed to see whether cancer cells differ in any of the post-translational modifications.

Determination of the IC 50 values of different antitumor drugs against human breast cancer cell lines MCF-7 and MDA-MB-231:

The anti-tumor activity of various drugs were tested against two human breast cancer cell lines, MCF-7 and MDA-MB-231. The former one is not metastatic, while

the latter is. The cells (10^4) were plated in a 96-well culture plate. After 24 h different concentrations of drugs were added in the culture medium and the cells were allowed to grow for 4-5 days. The cells were then trypsinized and counted in a hemacytometer using trypan blue dye exclusion method. The results are shown in Fig 2. and Fig. 3. As shown in Fig 2, for non-metastatic MCF-7 cells, maytansine is the most potent of all the drugs tested, with an IC_{50} value of around 0.1 nM. Maytansine is also found to be the most potent when tested against metastatic cell line MDA-MB-231(Fig. 3). Future experiments will be performed to test other drugs. Studies will also be initiated to isolate drug-resistant cells and identify possible alterations in the drug-binding site on tubulin.

KEY RESEARCH ACCOMPLISHMENTS AND CONCLUSIONS

- We have studied the interaction of two thicolchicine analogues with pure tubulin isoforms from brain and found significant differences in the affinity for different tubulin isoforms.

- We have purified tubulin from the breast cancer cells by paclitaxel-induced assembly.

- We have studied the post-translational tyrosination of tubulin in the breast cancer cells and find that tubulin from breast cancer cells is much more tyrosinated than brain tubulin.

- Immunoblotting studies with anti-β_{II} monoclonal antibody reveals that tubulin from the breast cancer cells lacks the β_{II} isoform of tubulin completely.

- Among the antitumor drugs tested, maytansine is the most potent of all the drugs in inhibiting the growth of breast cancer cells, followed by podophyllotoxin and paclitaxel.

REPORTABLE OUTCOMES

Abstracts presented in the 38th Annual Meeting of the American society for Cell Biology in san Francisco, CA, (December 12-16, 1998).
The abstracts are published in:

1. Mol. Biol. Cell 9, p149a. Ab#864
2. Mol. Biol. Cell 9, p150a. Ab#869
3. Mol. Biol. Cell 9, p409a. Ab# 2374

Published papers:

1. Banerjee, A., Kasmala, L. T., Hamel, E., Sun, L., and Lee, K.-H. (1999) Interaction of novel thiocolchicine analogs with the tubulin isoforms from bovine brain.
Biochem. Biophys. Res. Commun. **254**, 334-337

2. Banerjee, A. (1999) A monoclonal antibody to α–tubulin: purification of functionally active α–tubulin isoforms.
Biochemistry , 38, 5438-5446, April 27, 1999.

APPENDICES

List: Figures 1-3, Figure legends , 3 abstracts, 2 reprints of published papers.

Figure legends

Figure 1: Immunoblotting of breast cancer tubulin with anti-α tubulin antibody

Breast cancer cell extracts were incubated with 10 μM paclitaxel in the presence of 2 mM PMSF and protease inhibitors at 37^0C for 30 min. The assembled microtubules (MTs) were harvested by centrifugation at 100,000 g for 1 h at 25^0C. The microtubule pellets were dissolved in Laemmli sample buffer, boiled for 5 min and were subjected to SDS polyacrylamide gel electrophoresis. An identical gel was transferred onto a nitrocellulose membrane and was processed for immunoblotting with the monoclonal antibodies AYN.6D10, which is specific for tyrosinated α–tubulin and anti -β_{II} antibody that recognizes the β_{II} tubulin isoform. About 5 μg protein was loaded in each lane. The samples are: Lane 1, bovine brain PC-tubulin; lanes 2&4, Mt pellet from MCF-7; lane 3&5, supernatant from MCF-7 cells after harvesting of MTs; lane 6, Mt pellet from MDA-MB -231 cells; lane 7, supernatant from from MDA-MB -231 cells after harvesting of MTs. Note that the breast cancer α–tubulin is much more tyrosinated than brain α–tubulin, while β_{II} tubulin, which is the major isoform in brain, is completely absent from the breast cancer cells.

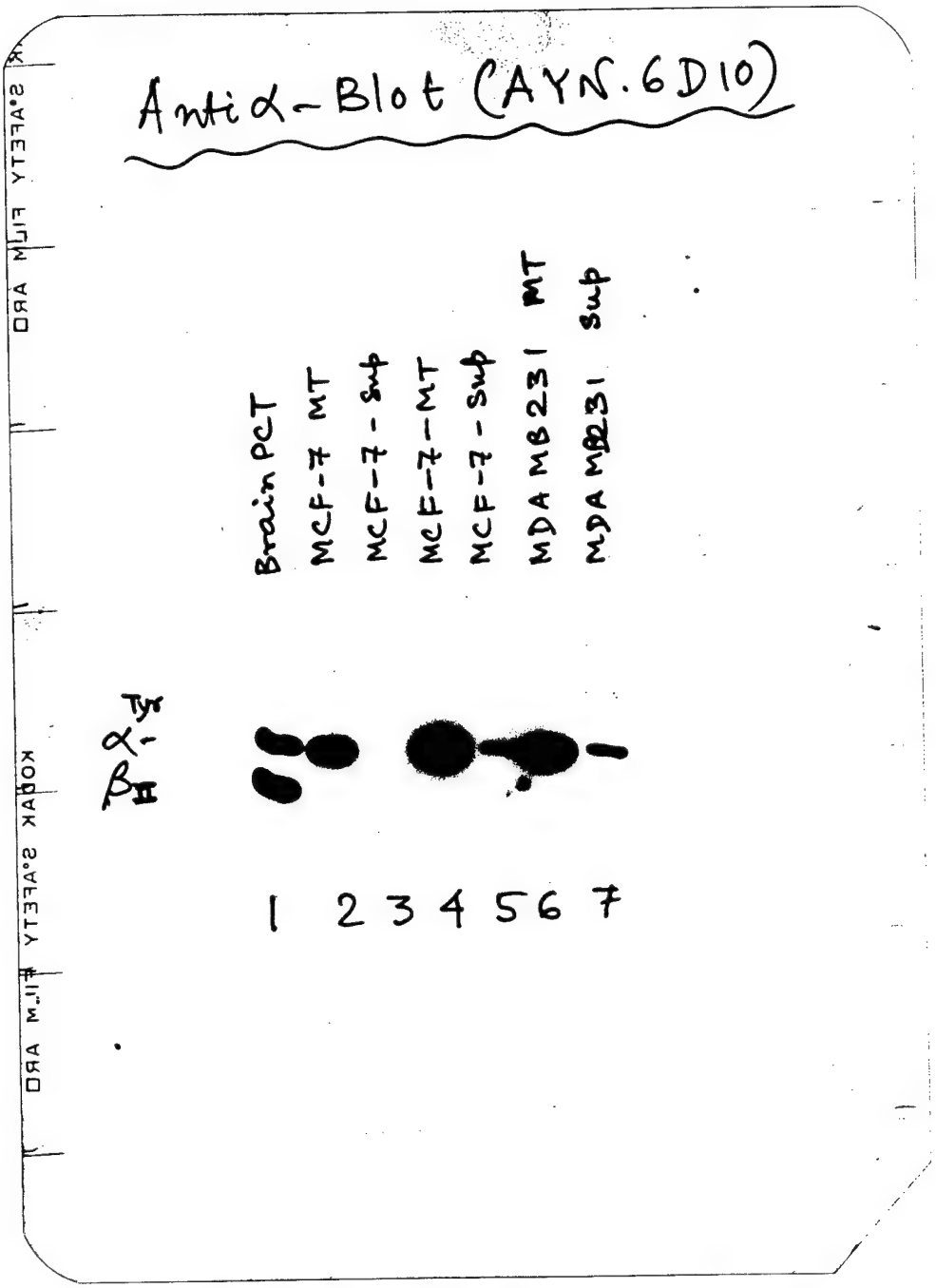
Figure 2 : Inhibition of the growth of MCF-7 breast cancer cells in the presence of antitumor drugs

The cells (10^4) were plated on a 96-well culture plates for 24 h and were incubated with different concentrations of drugs in culture medium. After growth for 4-5 days, the cells were trypsinized, resuspended and counted using trypan blue dye exclusion method. Curve 1, colchicine; curve 2, vinblastine, curve 3, paclitaxel; curve 4, podophyllotoxin, and curve 5, maytansine.

Figure 3 : Inhibition of the growth of MDA-MB-231 breast cancer cells in the presence of antitumor drugs

The cells (10^4) were plated on a 96-well culture plates for 24 h and were incubated with different concentrations of drugs in culture medium. After growth for 4-5 days, the cells were trypsinized, resuspended and counted using trypan blue dye exclusion method. Curve 1, podophyllotoxin; curve 2, colchicine, curve 3, vinblastine, and curve 4, maytansine.

FIGURE 1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Brain PCT
MCF-7 MT
MCF-7 - Sup
MCF-7-MT
MCF-7 - Sup
MDA MB231 MT
MDA MB231 Sup
α-Tyr
β-II
1 2 3 4 5 6 7

Fig 2: Effect of antitumor drugs on the growth of MCF-7 breast cancer cells

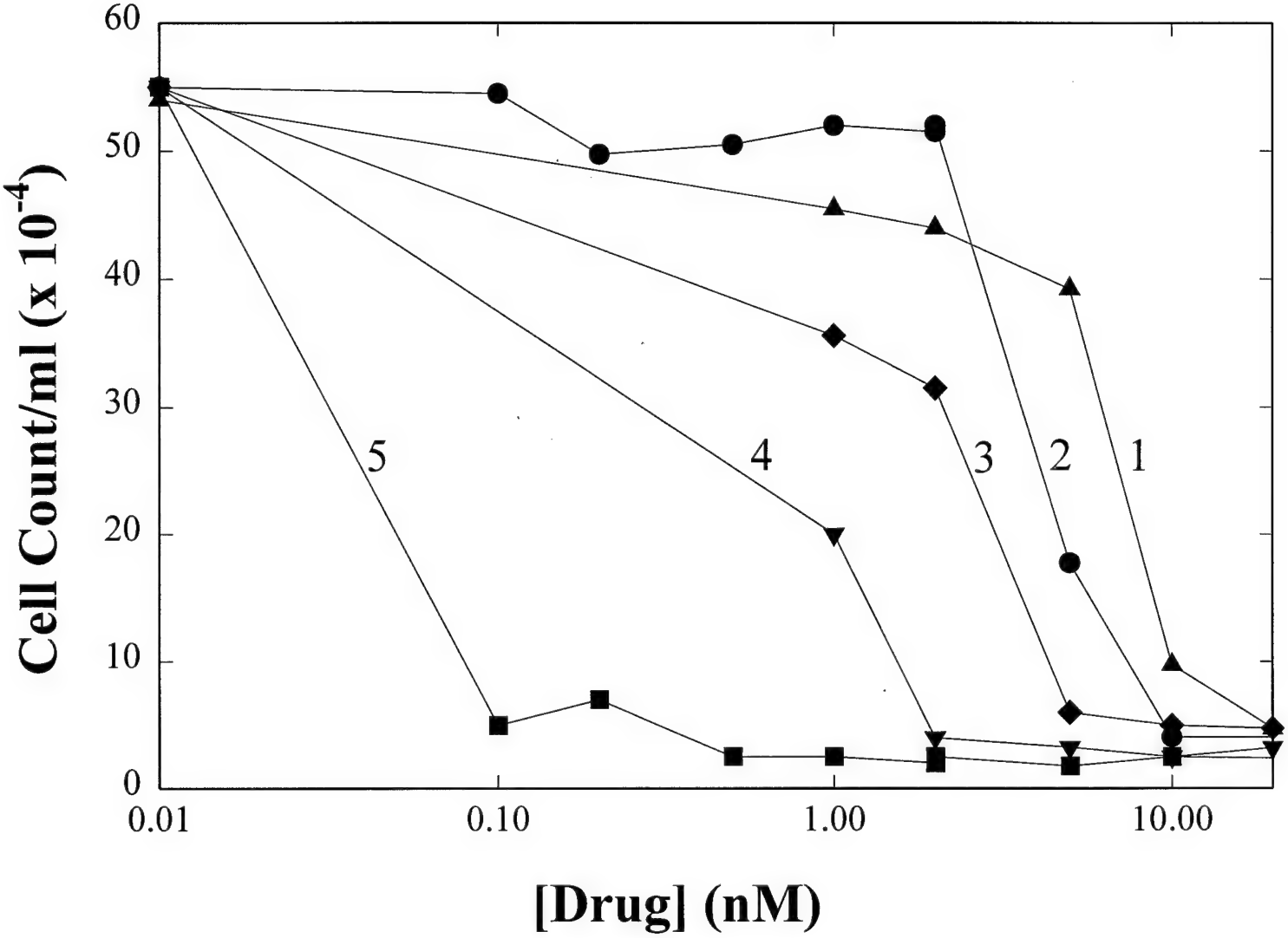
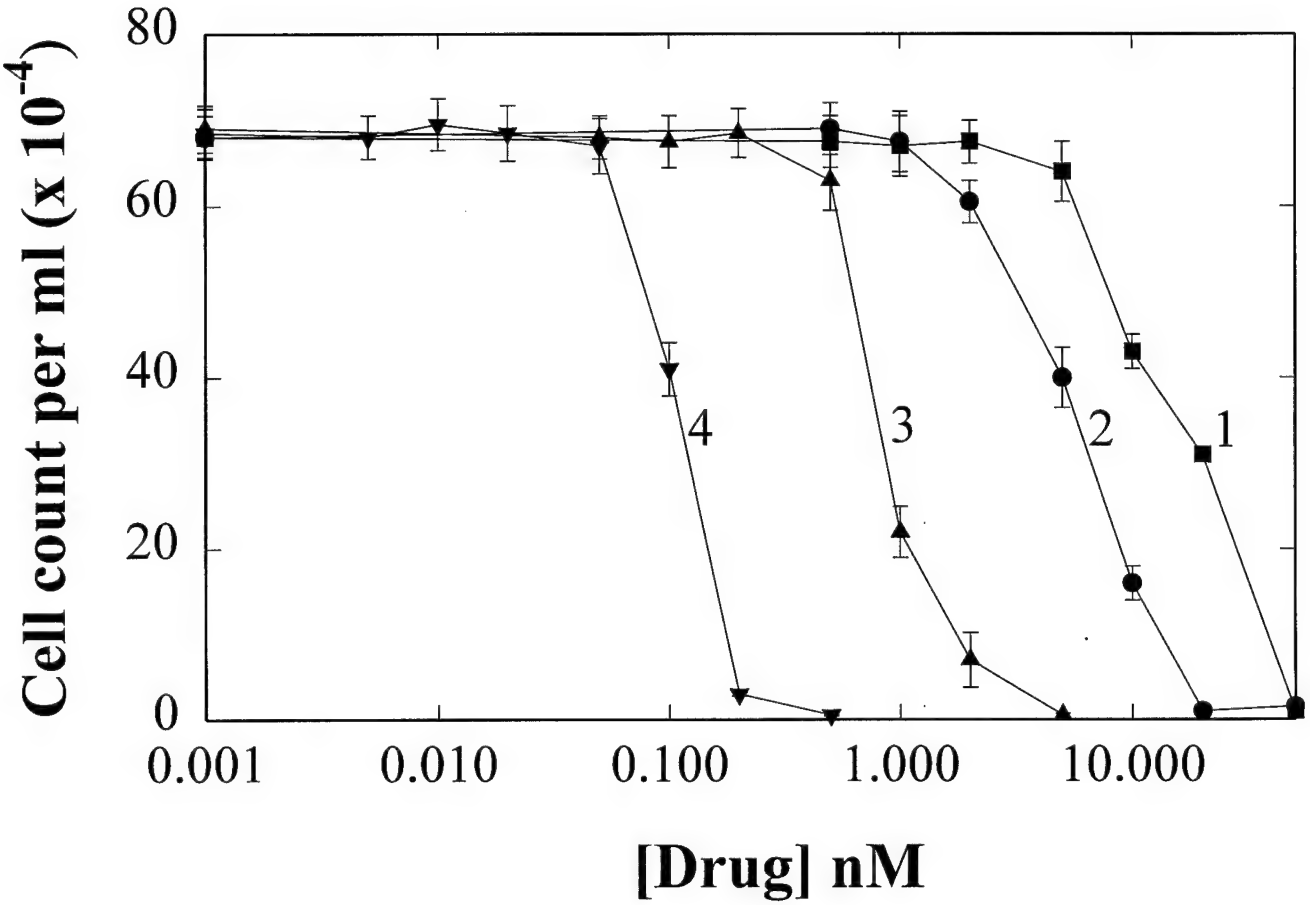


Fig. 3: Effect of antitumor drugs on the growth of MDA-MB -231 breast cancer cells



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CHIMERIC AXOLOTLS PRODUCE EMBRYOS WITH 100% CARDIAC MUTANT PHENOTYPE. Larry F. Lemanski, Masako Nakatsugawa, Sharon L. Lemanski, Robert Zajdel, Kent Salsbury, Matthew McLean and Dipak K. Dube. Department of Medical Physiology, Texas A and M University, College Station, TX 77843 and Department of Anatomy and Cell Biology, SUNY Health Science Center, Syracuse, NY 13210.

We have used the cardiac mutant axolotl system as a unique model to study vertebrate heart development. Simple recessive gene c, in homozygous condition, results in 25% of the offspring forming hearts which lack myofibrils and fail to beat. Thus, in past studies it was not possible to distinguish normal from mutant siblings until stage 34, (late tailbud stage), when the hearts began to contract in normals. As a consequence, it was not possible to efficiently examine pre-heartbeat stages of known mutant embryos or to design and execute experiments on gene expression as well as gene manipulation at early pre-heartbeat stages. We recently have overcome this problem by creating chimeric animals composed of normal (+/+) head heart regions grafted onto the bodies of mutant (c/c) embryos, which develop mutant (c/c) gonads. Matings of such chimeras produce 100% known c/c mutant offspring which can be used a in experiments from the fertilized egg onward. We have verified that embryos from such spawnings are 100% mutant by using established criteria for determining the mutant phenotype, including, the absence of beating hearts and gil circulation, lack of organized myofibrils by electron microscopy and lack of normal myofibril staining patterns by immunofluorescent microscopy after antitropomyosin staining. These chimeras open up an exciting new area of research by permitting various cellular and molecular studies, including gene manipulation and gene therapy experiments, on known mutant embryos at the earliest stages of development. (Supported by NIH HL 58435, NIH HL 061246 and an AHA Grant.)

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EXPRESSION OF A NOVEL ISOFORM OF TROPOMYOSIN IN STRIATED MUSCLES OF NORMAL AND CARDIAC MUTANT *MEXICAN AXOLOTLS.* D.K. Dube[1], R.W. Zajdel[1], B.J. Spinner[1], M.D. McLean[1], S. Dube[2] and L.F. Lemanski[3]. [1]Department of Anatomy & Cell Biology, SUNY Health Science Center, Syracuse, NY 13210. [2]Department of Medicine, SUNY HSC. [3]Department of Medical Physiology, Texas A and M University, TX 77843.

The cardiac lethal mutation in the Mexican axolotl, Ambystoma mexicanum, has proven a useful tool for studying heart development in vertebrates because it carries a simple homozygous recessive mutation that results in a failure of the affected hearts to beat. Immunohistochemical and ultrastructural analyses demonstrate that the mutant hearts do not beat because they are deficient in tropomyosin and lack organized myofibrils. We have recently reported the presence of a novel isoform of tropomyosin, ATmC-2, in the axolotl heart. ATmC-2 is most likely an alternatively spliced product of the same α-TM gene which also encodes ATmC-1, a typical striated muscle specific tropomyosin isoform. In ATmC-2 the amino acid sequence of exon 2 is comparable to vertebrate smooth muscle type tropomyosin. The level of transcript of ATmC-2 was found to be predominant in cardiac tissues in adult axolotl whereas the transcript level of ATmC-1 was predominant in skeletal muscles. We have raised ATmC-2 isoform-specific anti-peptide antibody in rabbits and subsequent studies with C2 antibody reveal that ATmC-2 transcripts are translated both in cardiac and skeletal muscles; also it is localized in the myofibrils. C-2 antibody does not recognize mammalian and avian α-tropomyosins. Although immunohistochemical staining with C2 and anti-α tropomyosin antibody (CH-1) are different in cardiac tissues, the staining patterns of skeletal muscle with these two antibodies appear virtually identical. Another striking observation is that the levels of ATmC-2 and ATmC-1 protein in skeletal muscle are comparable although the level of ATmC-2 transcript is significantly lower in skeletal muscles. (Supported by NIH HL 58435, HL 061246 and an AHA Grant.)

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CELL SHAPE DETERMINES THE FATE OF SMOOTH MUSCLE CELL PRECURSORS Yan Yang, Jianbo Liu and Lucia Schuger. Department of Pathology, Wayne State University School of Medicine, Detroit, MI 48201

We found that the mesenchymal cells from embryonic mouse organs are optative smooth muscle (SM) cell precursors. We isolated embryonic mesenchymal cells from muscular (intestine), non-muscular (kidney), or partially muscular (lung) organs prior to the onset of SM differentiation and cultured them under conditions that promoted either cell rounding or cell spreading/elongation. Early embryonic mesenchymal cells are normally round in vivo and cell rounding was maintained in culture by plating the cells at confluent densities, or by plating them on 0.1% poly-L-lysine. Cell spreading/elongation was allowed by plating the cells at sub-confluent densities, or by plating them on 0.01% poly-L-lysine. Spread/elongated cells differentiated into SM regardless of the organ of origin, as indicated by the expression of SM-myosin, SM-α actin, SM-γ actin, calponin, SM-22 and desmin. SM differentiation occurred in less than 24 hours, involved all the cells in the culture, and was independent of cell proliferation. The round cells, on the other hand, remained negative for SM markers, but were able to differentiate into SM when transferred to culture conditions that facilitated cell spreading. Cell shape overdid the normal differentiation pathway of the cells in vivo, as indicated by the absence of SM differentiation in rounded intestinal cells and its occurrence in spread kidney cells. Our studies indicated that cell shape also overrides the effect of retinoic acid, TGF-β, PDGF and epithelial/endothelial-mesenchymal cell interactions, all which have been shown to stimulate SM differentiation. This work was supported by NIH grant Hl48730.

Tubulin I (864-865)

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DO α– AND β–TUBULIN PAIR MONOGAMOUSLY ?((A. Banerjee, L. T. Kasmala, R. F. Ludueña)) Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX 78284-7760. (Spon. by M. Venkatachalam)

Mammalian α- and β-tubulin occur as various isoforms that may originate from different primary sequences as well as their post-translational modifications. It is not clear how an individual α–tubulin pairs with another β–tubulin. To address this question, we have purified tubulin heterodimers with a single class of α-tubulin using an immunoaffinity column specific for α–tubulin. Four fractions A, B, C, and D were obtained. Fraction A is the Δ2 form of Mα1/2, fraction B is a mixture of the detyrosinated forms of Mα1/2 and Mα4, fraction C is predominantly the tyrosinated form of Mα1/2, and the fraction D is the tyrosinated form of Mα4. The composition of different βs associated with each α was determined by SDS-PAGE followed by immunoblotting with monoclonal antibodies specific for different β–tubulin classes. Unfractionated bovine brain tubulin contains 3% β_I, 58 % β_{II}, 25 % β_{III}, and 13 % β_{IV}. In contrast, analysis of the β–tubulin content in each fraction shows that tyrosinated Mα1/2 contains 2-3 fold higher amounts of β_{III} than does tyrosinated Mα4, while the latter contains 2-3 fold higher amounts of β_{IV} than that of the former. The amount of β_{II} associated with the Δ2 form of Mα1/2 was 2-3 fold lower than that of unfractionated tubulin. The results indicate that although tubulin monomers can pair non-specifically, the affinity for any particular αβ combination may differ significantly. (Supported by grants CA59711 from the NIH and DAMD 17-98-1-8244 from the US Army to AB, and CA 26376 from the NIH and Welch grant AQ-0726 to RFL).

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TUBULIN-GDP POLYMERIZES INTO MICROTUBULES IN THE PRESENCE OF THE NATURAL OSMOLYTE TRIMETHYLAMINE-N-OXIDE (TMAO). ((Rita D. Ward and Dan L. Sackett)) Laboratory of Neurobiology, NINDS, and Laboratory of Drug Discovery Research and Development, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health, Bethesda, MD

TMAO is a natural osmolyte that protects native folded structures against destabilizing agents such as urea. It was previously shown that TMAO promotes microtubule (MT) polymerization from tubulin-GTP, largely reverses urea inhibition of polymerization, and does not interfere with binding of MAPs (Am. J. Physiol. 273: R669-R676, 1997). MT polymerized in 1 M TMAO are fully cold sensitive and appear normal in electron microscopy studies. At 1.5 M TMAO or above, MT are hyperstable and are not depolymerized by cold. In addition, non-MT structures, notably curved, protofilament-like polymers, are also seen. The stabilization against cold is similar to the effect of Taxol, and like Taxol, TMAO can induce MT polymerization from tubulin-GDP. This requires approximately 0.25 M more TMAO than with tubulin-GTP. At 1.25 M TMAO, tubulin-GDP readily polymerizes into MT that appear normal in negative stain electron micrographs. The yield of polymer is similar to that obtained at 1 M TMAO with tubulin-GTP. At 1.75 M TMAO or above, polymerization of tubulin-GDP yields shorter MT than at lower TMAO concentrations. In addition, the non-MT, protofilament-like polymers observed at 1.5 M TMAO with tubulin-GTP are observed.

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INTRINSIC DISULFIDE BONDS IN TUBULIN REGULATE FOLDING AND STABILITY *IN VITRO* ((A. R. Chaudhuri, I. A. Khan and R. F. Luduena)) Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78284-7760. (Spon. by I. A. Khan.)

The sulfhydryl groups of tubulin are actively involved in regulating ligand interactions and microtubule formation both *in vivo* and *in vitro*. Although tubulin has twenty cysteine residues in its primary structure, it is not clear how many of these are normally oxidised in disulfides. We have investigated the presence of disulfide bond(s) in tubulin. We denatured phosphocellulose-purified and isotypically purified tubulin and added cold iodoacetamide. Then we removed the unreacted iodoacetamide. We then added dithiothreitol (DTT) followed by iodo[^{14}C]acetamide and measured incorporation of ^{14}C. We found that the samples which were not treated with DTT had no incorporation of iodo[^{14}C]acetamide while the others which were treated with DTT had significant amounts of incorporation. Interestingly, isotypically purified αβIV tubulin appears to have an extra disulfide bond not present in αβII and αβIII. We also found that these disulfide linkages regulate the folding and the stability of the protein *in vitro*. At present, we are actively involved in identifying the cysteines that are involved in disulfide bond formation. (Supported by NIH grant CA26376 and Welch grant AQ-0726 to R.F.L.)

867

DISSECTING THE MOLECULAR BASIS OF THE CONFORMATIONAL CHANGE UNDERLYING THE DYNAMIC PROPERTIES OF ASSEMBLED MICROTUBULES. ((R. G. Burns)) Biophysics Section, Blackett Laboratory, Imperial College of Science, Technology and Medicine, London SW7 2BZ.

Direct and indirect experimental evidence strongly suggests that β-tubulin, which is exposed at the (+)end of the assembled microtubule, undergoes one or more conformational changes in response to the assembly-dependent GTP hydrolysis, and that such change(s) facilitate dynamic instability and treadmilling. Consideration of the dynamic properties of the (-)end suggests that the α-subunit must also undergo conformational change(s), possibly by relaxing to a lower energetic state. Similarly, hydrolysis-induced change(s) in the β-subunit may be transmitted via an α-subunit to other β-tubulin subunits. The molecular basis of the change(s) in the α– and β–tubulins are likely to be related and to be highly conserved. Inspection of a database consisting of 195 α– and 242 β– full-length or partial tubulin sequences reveals that a remarkable fraction of the residues are extraordinarily highly conserved. In particular, 143 α– and 162 β–tubulin residues are conserved in at least 98% of the available sequences, of which 95 of the positions are conserved in both subunits. These values increase to 223 and 235 residues (with 156 at common positions) when applying a 95% conservation level. The residues which are conserved in both subunits have been mapped to the low-resolution tubulin tertiary structure (Nogales and Downing, 1998). This reveals that the conserved residues are predominantly confined to peptides associated with either the nucleotide-binding sites or with the longitudinal inter-monomer interfaces. By contrast, residues contributing to the lateral inter-protofilament interfaces are less highly conserved. It is proposed that the mechanism which effects the conformational change(s) depends primarily on the residues defining the longitudinal interactions, and that these changes lead directly to the observed effects on the association and dissociation rate constants.

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POSTTRANSLATIONAL GLUTAMYLATION OF BRAIN TUBULINS FROM THE ANTARCTIC FISH *NOTOTHENIA CORIICEPS*. ((V. Redeker[1], A. Frankfurter[2], J. Rossier[1], H.W. Detrich, III3[3])) [1]Lab. Neurobiologie, CNRS UMR 7637, 10 r. Vauquelin, 75005 Paris, France; [2]Dept. Biology, Univ. of Virginia, Charlottesville, VA 22901; [3]Dept. Biology, Northeastern Univ., Boston, MA 02115

We have shown previously that the microtubules of Antarctic fish assemble efficiently at low temperatures (-2 to +2°C) due to adaptations intrinsic to the tubulin subunits. To determine whether changes in posttranslational glutamylation of the fish tubulins (addition of several glutamates to the side chain of one or more glutamate residues in the carboxyl termini of the tubulin chains) may contribute to cold adaptation of microtubule assembly, we have characterized C-terminal peptides from brain tubulin chains of the Antarctic rockcod *Notothenia coriiceps* by MALDI-TOF mass spectrometry and by amino acid sequencing. To assist interpretation of the MALDI-TOF spectra the primary sequences of the C-termini of six α-tubulins and three β-tubulins from *N. coriiceps* were deduced by sequencing the 3' ends of brain tubulin cDNAs. For the β-tubulin isotypes, non-glutamylated isoforms are apparently more abundant than glutamylated isoforms. In addition, maximal glutamate chain length is less than that observed for mammalian brain tubulins. For the α-tubulin isotypes identified by protein chemistry, in contrast, both the proportion of glutamylated forms and the maximal glutamate chain length generally exceed those observed for adult rat brain tubulin. Thus, Antarctic fish brain tubulins are glutamylated differently than mammalian brain tubulins, yielding an increase in heterogeneity of α isoforms and a reduction in heterogeneity of β isoforms. We suggest that unique residue substitutions in the primary sequences of Antarctic fish tubulin isotypes and quantitative changes in isoform glutamylation act synergistically to adapt microtubule assembly to low temperatures. Supported by grants: ARC 9343 (VR,JR), NIH NS21142 (AF), NSF OPP-9120311 and OPP-9420712 (HWD).

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DO DIFFERENT POST-TRANSLATIONAL MODIFICATIONS IN TUBULIN REGULATE MICROTUBULE ASSEMBLY ? ((A. Banerjee and L. T. Kasmala)) Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX.

Both α- and β-tubulin exist as numerous isotypic forms that may originate from different primary sequences as well as their post-translational modifications. It is not clear whether different tubulin isoforms or their post-translational modifications affect functional properties of tubulin. In an effort to understand the role of different α-tubulin isoforms, a monoclonal antibody, AYN.6D10, was prepared against the mammalian α-tubulin carboxy terminal sequence Glu-Glu-Gly-Glu-Glu-Tyr. By using the immunoaffinity column, bovine brain tubulin was fractionated into three functionally active αβ heterodimers containing a single α-tubulin class. The isoforms were identified by immunobloting with α–tubulin specific antibodies and sequence analysis. Three out of four isoforms are functionally active as judged by their colchicine-binding activity. Assembly studies in the presence of glycerol and Mg^{++} show that one of these isoforms, identified as the tyrosinated Mα1/2 isoform, assembled poorly, while its Δ2 form assembled normally. Mass spectrometric studies revealed that the tyrosinated Mα1/2 differs from its Δ2 form in the post-translational modification. The Δ2 form of Mα1/2 predominantly contains a side chain of 1-2 glycyl units with no glutamyl side chain, while the tyrosinated Mα1/2 contains a side chain of 1-4 glutamyl units with no polyglycyl side chain. Future studies with the isoforms may yield valuable information regarding the roles of polyglutamylation and polyglycylation in in regulating microtubule assembly and function *in vivo*. (Supported by grants CA59711 from the NIH and DAMD 17-98-1-8244 from the US Army to AB, and NIH grant CA 26376 and Welch grant AQ-0726 to RFL).

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MUTATION OF CONSERVED CYSTEINES IN β-TUBULIN. ((M.L. Gupta[†], C.A. Dougherty[‡], and R.H. Himes[†])) [†]Dept. of Biochem., Cell and Mol. Biol., Univ. of Kansas, Lawrence, KS 66045 and [‡]Dept. of Biol., Johns Hopkins Univ., Baltimore, MD 21218.

It is well known that the biological activity of tubulin is sensitive to sulfhydryl modifying reagents. To investigate the importance of specific cysteine residues in tubulin function, we have begun to mutate the conserved cysteines in the yeast *Saccharomyces cerevisiae* α- and β-tubulins. Here we present the phenotypes of cells which contain both alanine and serine substitutions at the five conserved cysteines in β-tubulin (Cys 12, 127, 201, 211, 354). Cells with substitutions at Cys127 have normal growth characteristics and sensitivity to the anti-microtubule drug benomyl, while Cys201 and Cys211 mutants were wild-type for growth and slightly benomyl sensitive. In contrast, we see more dramatic phenotypes in cells that contain Cys12 or Cys354 mutations. These mutants are slow growing; apparently due to delays in the G2/M phase of the cell cycle, as we have measured an increase, versus the control strain, in the percentage of large-budded cells, average cell size and cells with 2N DNA content in logarithmically growing cultures. The growth defect is more severe in Cys354 mutants which display a variety of aberrant cell morphologies including large rounded cells, elongated buds, elongated neck regions and multibudded cells. Cys354 mutants also display decreased sensitivity to benomyl. The mitotic defects of the Cys12 and Cys354 mutants are consistent with these residues being important for microtubule function. Indeed, both of these residues have been shown by cross-linking studies to be at or near domains involved in modulating tubulin function, Cys12 at the GTP-binding pocket and Cys354 at the colchicine binding site. We are currently purifying the mutated tubulins using a 6xHis affinity tag at the C-terminus of β-tubulin and will determine the contribution of each cysteine toward tubulin's biochemical properties. Supported by a grant from the University of Kansas.

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POLYGLYCYLATION AND POLYGLUTAMYLATION ARE MULTIPLE SITES POSTTRANSLATIONAL MODIFICATIONS OF TUBULIN J. Vinh(1), V. Redeker(1). A. Frankfurter(2), M.-H. Bré(3), N. Levilliers(3) & J. Rossier(1). (1) Lab. de Neurobiologie, ESPCI-CNRS UMR7637, 10 rue Vauquelin, 75231 PARIS Cedex 05. France (2) Dept. Of Biology, Univ. of Virginia, Charlottesville, VA22901. U.S.A. (3) Lab. de Biologie Cellulaire. CNRS URA2227, Univ. Paris-Sud, 91405 ORSAY Cedex, France

Polyglycylation and polyglutamylation are major posttranslational modifications specific to tubulin. They are localized in the carboxy terminal isotype-specific domain of both α- and β-tubulin. Polyglycylation and polyglutamylation involve the formation of lateral polyglycine or polyglutamate chains respectively covalently bound on the γ-carboxyl group of at least one glutamate residue in the backbone sequence. Both of them occur in numerous species, from monocellular protists to mammals. In this work, Edman degradation and mass spectrometry sequencing have been used to demonstrate that one single tubulin isotype can be simultaneously modified on multiple sites. Paramecium β-tubulin polyglycylation occurs simultaneously on four sites and exhibits one major hexaglycylated structure (two glycine residues on both Glu^{437} and Glu^{438}, and one glycine residue on both Glu^{439} and Glu^{441} in the C-terminal sequence ^{427}DATAEEEGEFEEEGEQ^{442}). Adult rat brain α4-tubulin polyglutamylation occurs on two sites (Glu^{443} and Glu^{445} in the C-terminal sequence ^{431}DYEEVGIDSYEDEDEGEE^{448}). A complete description of the structural characterization procedure using Post Source Decay Matrix-assisted Laser Desorption/Ionization mass spectrometry (PSD MALDI MS), Electrospray Ionization Quadrupole Time-of-flight hybrid tandem mass spectrometry (ESI Q-Tof MS/MS) and Edman degradation sequencing will be presented.

2370

THE ROLE OF DELTA-TUBULIN AND THE C-TUBULE IN BASAL BODIES. Sylvia Fromherz[1], Ursula W. Goodenough[2], Thomas Giddings[1], Andrea M. Preble[1], and Susan K. Dutcher[1]. [1]MCD Biology. University of Colorado. Boulder. CO [2]Department of Biology. Washington University, St. Louis. MO

Delta (δ)-tubulin is the fourth member of the tubulin superfamily and is equally distant phylogenetically from α-, β-, and γ-tubulin. A deletion of this gene in *Chlamydomonas* results in viable cells that have a heterogeneous flagellar assembly defect. When cells were examined by electron microscopy. doublet rather than triplet microtubules were observed in the basal bodies (Dutcher and Trabuco. 1998). The C-tubule is missing. Basal bodies with doublet microtubules are located in the proper cellular position and associate correctly with cytoplasmic striated fibers and microtubules. Immunofluoresence with polyclonal antibodies to the N-terminus of δ-tubulin suggests that it is predominantly localized to basal bodies in *Chlamydomonas* and in human sperm. The *Chlamydomonas* and human δ-tubulin homologs are 43% identical at the amino acid sequence level. Suppressors of the flagellar assembly defect were isolated. Two different missense mutations (D205N and A208T) in the α2-tubulin gene completely suppress the flagellar assembly defect and restore triplet microtubules. However, the triplet microtubules in the double mutant cells do not have a normal morphology. Both the aspartate and the alanine residues are conserved in 383 of 384 α-tubulin sequences in GenBank. These α2-tubulin mutations in the absence of the δ-tubulin mutation confer hypersensitivity to colchicine. We suggest that the D205N and A208T mutations may mediate changes in the microtubule lattice that allow for the formation of triplet microtubules in the absence of δ-tubulin.

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THE NUCLEOSIDE TRIPHOSPHATE SPECIFICITY OF TUBULIN. ((G. Chakrabarti, M.R. Mejillano, Y.H. Park, and R.H. Himes)) Department of Biochemistry, Cell and Molecular Biology, University. of Kansas, Lawrence, KS 66045.

We are interested in the interactions between tubulin and GTP that are responsible for the specificity shown for the nucleoside triphosphate. To achieve this aim we have determined the binding affinities of the four purine nucleoside triphosphates, GTP, ITP, XTP and ATP, and two γ-phosphoryl modified analogues, p-azidoanilido-GTP and m-acetylanilido-GTP. MgGTP, with a K_s of $5x10^7$ M^{-1} binds with an affinity that is 300-fold higher than that for MgITP, 1400-fold higher than for MgXTP, and 3000-fold higher than that for MgATP. The major effect on binding is the removal the 2-exocyclic amino group from the purine ring, suggesting that this function H-bonds to a group in the protein, either directly or through an intervening H_2O molecule. In comparing the affinities of ITP and ATP it is clear that the 6-oxo function of GTP also makes a contribution to the binding energy. The γ-phosphoryl-modified analogues act as inhibitors of assembly. Their binding affinities are higher than that for ITP, suggesting that modifications at the γ-phosphoryl group do not affect binding as much as modifications in the purine ring. Inhibition by γ-phosphoryl analogues modified with a bulky group is consistent with the structure of tubulin that shows that the E-site region of β-tubulin interacts with the α-subunit of another dimer.

2372

A system for high through-put screening for tubulin and microtubule ligands.
Davis, A. and Middleton, K.M. Cytoskeleton, Inc. 1650 Fillmore Street, #240, Denver, CO 80206. USA.

Tubulin and microtubules are targets for a multitude of anti-tumor compounds. Tubulin polymerization can be measured by an increase in optical density over time. Previously this assay has been used on a small scale to characterize cytotoxic compounds and to screen derivative of a parent compound. The assay has been not been applicable to medium or high through-put assays before now. We present a system that is capable of screening the activity of a random library of compounds for tubulin ligands. The system is tested in 96- and 384-well format and it is suitable for screening upward of 100 000 compounds within one month. The present coefficient of variation is 16, 13 and 10% for single, duplicate and triplicate assays respectively. This research was sponsored by the NIH Small Business Innovative Research fund, GM53696-03.

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INTERACTION OF FHIT AND TUBULIN PROTEINS. ((A. R. Chaudhuri, I. A. Khan, V. Prasad, A. K. Robinson, R. F. Luduena, and L. D. Barnes)) Department of Biochemistry, Univ. Texas Health Science Center, San Antonio, TX 78284-7760

FHIT is a candidate human tumor suppressor gene. Homozygous deletions in the *FHIT* locus have been observed in numerous tumor-derived cell lines and aberrant transcripts have been observed in different types of primary tumors. Fhit is a dinucleoside 5'-oligophosphate hydrolase with Ap_3A as the preferred substrate. Homogeneous Fhit has been covalently modified with 6-iodoacetamidofluorescein to examine its potential interaction with tubulin by fluorescence spectroscopy. Fhit interacts with phosphocellulose-purified bovine brain tubulin in a concentration-dependent manner with a K_d of 1.4 μM. Cleavage of the C-terminus of tubulin with substilisin decreased its affinity for Fhit as indicated by a K_d of 4.1 μM. The interaction of Fhit with isotypic forms of β-tubulin yielded K_d values of 3.9 μM, 0.1 μM and 4.8 μM for $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$ tubulins, respectively. Fhit alone does not promote the assembly of tubulin into microtubules, but it markedly increases the extent of assembly in the presence of tau, and the microtubules formed exhibited normal structures as detected by electron microscopy. Fhit is unusual in being a tubulin ligand with a strong interaction with $\alpha\beta_{III}$-tubulin. This interaction and the effect of Fhit on microtubule formation may be involved in the mechanism of Fhit as a tumor suppressor. (Supported by NIH grant CA26376 and Welch grant AQ-0726 to RFL and NSF grant MCB-9604124 to LDB.)

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INTERACTION OF NOVEL THIOCOLCHICINE ANALOGS WITH BOVINE BRAIN TUBULIN((A. Banerjee[1], L. T. Kasmala[1], R. F. Luduena[1], E. Hamel[2], L. Sun[3]. and K.-H. Lee[3])) [1]Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX 78284-7760; [2] National Cancer Institute. NIH. Frederick. MD 27102; and [3] Natural Products Laboratory. University of North Carolina, Chapel Hill, NC 27599. (Spon. by P. Saikumar)

The antimitotic alkaloid colchicine binds to tubulin and inhibits microtubule assembly. Tubulin occurs as several isoforms in different tissues and species. Although colchicine does not have good anti-tumor properties. a new series of colchicine analogues have been prepared in which the middle seven-membered B-ring was replaced by a six-membered ring and the methoxy group in the tropolone ring was replaced by a thiomethoxy group. Several compounds of this series were found to exhibit differential antitumor properties in certain solid tumor cell lines. In an effort to examine whether the differential reactivity of the analogs are correlated with their interactions with different tubulin isoforms, we have studied the interaction of two analogs with unfractionated bovine brain tubulin and two β-tubulin isoforms $\alpha\beta_{II}$ and $\alpha\beta_{III}$. The interaction was studied by measuring the quenching of intrinsic tryptophan fluorescence of tubulin. We find that one analog THC5 which contains a -CH_2OH group in its B-ring, does not exhibit differential interaction with different tubulin isoforms. In contrast, when the side chain was reduced to $=CH_2$, the analog THC18 exhibited differential interaction with the tubulin isoforms. The affinity constants for $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ were 30-50 fold higher than that of $\alpha\beta_{III}$. The results indicate that the side chain on the B-ring may be very critical for the tissue-specificity of the drugs. Supported by grants CA59711 from the NIH and DAMD 17-98-1-8244 from the US Army to AB, and CA 26376 from the NIH to RFL).

2375

INTERACTION OF THE β_{IV}-TUBULIN ISOTYPE WITH ACTIN STRESS FIBERS IN CULTURED RAT KIDNEY MESANGIAL CELLS. ((C. Walss[1], V. Prasad[1], J.I. Kreisberg[2], and R.F. Luduena[1])) Departments of [1]Biochemistry and [2]Pathology, University of Texas Health Science Center, and [2]Research Service, Audie Murphy VA Hospital, San Antonio, TX 78284. (Spon. by S. Shain).

Glomerular mesangial cells are contractile cells which have been shown to undergo cAMP-mediated shape change in culture. Actin stress fiber disassembly occurs concomitantly with the shape change and an intact microtubule network is necessary for this to occur. In the present study, we used a double immunofluorescence labeling technique to investigate the interactions between the tubulin β-isotypes and the actin stress fiber network in cultured rat kidney mesangial cells. Removal of the soluble cytoplasmic and nucleoplasmic proteins by detergent extraction caused the microtubule network to disappear while the stress fiber network was still present. In these cells, the β_I- and β_{II}-tubulin isotypes were no longer present in the cytoplasm while the β_{IV} isotype was seen to interact with the actin cytoskeleton since the β_{IV} antibody staining co-localized with rhodamine phalloidin, which stains F-actin. This interaction between actin filaments and the β_{IV} isotype of tubulin raises the possibility that tubulin isotypes may have specific functions in the cell. (Supported by grants CA26376 from the N.I.H. and AQ-0726 from the Welch foundation to R.F.L. and a VA Merit Review to J.I.K)

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Interaction of Novel Thiocolchicine Analogs with the Tubulin Isoforms from Bovine Brain[1]

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The antimitotic alkaloid colchicine binds to tubulin and inhibits microtubule assembly. Recently a new series of colchicine derivatives has been synthesized in which the seven-membered B-ring was shortened to a six-membered ring. In an effort to study the role of the B-ring substituents in this new series, we have studied the interaction of two compounds of this series, THC 5 and THC 18, with tubulin isoforms from bovine brain. We find that THC 18, which has a side chain with a pi-bonded SP^2 conformation, binds differently to the tubulin isoforms, while THC 5 with a slightly different side chain does not. The results indicate that the conformation of the B-ring domain plays a major role in the differential interaction of a colchicine derivative with different tubulin isoforms. The results will be very important in designing potent antitumor derivatives of colchicine.

The antimitotic alkaloid colchicine binds to a single high-affinity site on the tubulin heterodimer and inhibits its assembly (1–4). Tubulin, the heterodimeric protein of microtubules occurs as different isoforms which are distributed differently in different tissues; some isoforms are prevalent in certain tissues while they occur in minor amounts in other tissues (5–8).

The binding of colchicine to tubulin is quite slow but stable, and results in conformational changes in both tubulin as well as colchicine (9–11). The binding results in an enhancement of drug fluorescence (12, 13), and results in the quenching of intrinsic protein fluorescence (14).

Numerous studies were initiated to determine the structural roles played by different parts of the colchicine molecule in its binding to tubulin. It has been suggested that the presence of both the ring-A and the ring-C are essential for the binding and minor structural changes in these two rings result in the loss of tubulin binding activity (4, 15). The middle ring-B plays a very important role in the binding; it is responsible for the slow and stable binding; a gradual shortening of the side-chain on this ring results in an increase in the binding rate and complete removal of this ring results in an analog that binds to tubulin very rapidly and reversibly (16–18).

Although colchicine and its high affinity analogs are good antimitotic agents, their use as anti-cancer agents were not very successful. Thus, a new series of colchicine derivatives has been synthesized in which the seven-membered B-ring of colchicine was replaced with a six-membered ring and the methoxy group in ring-C was replaced with the thiomethyl group (16). Several analogs of this series have been found to exhibit high potency against certain solid tumor cell lines, while they are insensitive to others (16). For example, THC 18 is about 1600-fold more potent than THC 5 in inhibiting the growth of RPMI 7951 melanoma cells (19). In this paper, we have studied the interactions of two analogs of this series. THC 5 and THC 18 with β-tubulin isoforms by studying the quenching of tryptophan fluorescence of tubulin.

EXPERIMENTAL PROCEDURES

Materials. Thiocolchicine analogs THC 5 and THC 18 were synthesized as described previously (19). All other chemicals were obtained as described previously (20).

Preparation of tubulin. Microtubules were purified from bovine brain cortex by a cycle of assembly and disassembly and tubulin was purified from microtubules by phosphocellulose chromatography as described elsewhere (21). The β-tubulin isoforms $\alpha\beta_{II}$ and $\alpha\beta_{III}$ were prepared from PC-tubulin by chromatography using anti-β_{II}, anti-β_{III}, and anti-β_{IV} immunoaffinity columns (22). The isoforms were stored frozen in aliquots at −80°C in buffer A (0.1 M Mes–Na, pH 6.4; 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 1 mM GTP) containing 8 M glycerol. Glycerol and GTP were removed before each

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[2] To whom correspondence should be addressed.

Abbreviations used: Mes, 2-[*N*-morpholino]ethane-sulfonic acid; PC-tubulin, tubulin purified by phosphocellulose chromatography.

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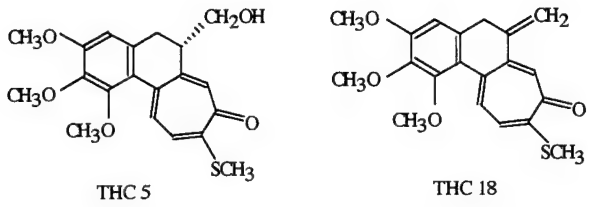

FIG. 1. Chemical structures of the thiocolchicine analogs.

experiment either by Sephadex G-25 column chromatography or by repeated centrifugation using Centricon 30 membrane filtration units. All isoform preparations were checked for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with isoform-specific antibodies.

Fluorescence measurements. The protein was incubated in the presence of drugs and the quenching of intrinsic tryptophan fluorescence was measured at 332 nm upon excitation at 278 nm. All fluorescence measurements were corrected for the inner-filter effects according to Lakowicz (23).

Analysis of fluorescence quenching data. The quenching of fluorescence was analyzed by a one- or two-affinity Michaelis–Menten binding model. The one-affinity model can be written as

$$Q = \frac{Q_m.D}{Kd + D},$$

where Q is the fluorescence quenching at any drug concentration D, and Q_m is the fluorescence quenching value at the saturating drug concentration,

$$Q = Q_1 + Q_2$$

where Q is the fluorescence quenching at any drug concentration D, and Q_1, Q_2 are the fluorescence quenching values for each class of complex,

$$Q_1 = \frac{Q_1.D}{Kd_1 + D}, \quad Q_2 = \frac{Q_2.D}{Kd_2 + D}.$$

where Kd_1 and Kd_2 are the dissociation constants for different class of complexes; Qm_1, and Qm_2 are the fluorescence quenching values for individual complexes at the saturating drug concentration. Each set of data was analyzed for one, or two-affinity model and the best-fit was judged by the analysis of residuals (plot of χ^2 versus D, where, χ = experimental data − fitted data). The best-fit was the one for which the sum of the square of residuals (χ^2) was minimum.

Data analysis. The raw data were analyzed by using a Packard Bell 486 personal computer and a curve-fitting software MINSQ obtained from MicroMath Software (Salt Lake City, UT).

RESULTS AND DISCUSSION

Quenching of intrinsic tryptophan fluorescence of tubulin by THC 5 and THC 18. The structures of the thiocolchicine analogs are shown in Fig. 1. THC 5 differs from THC 18 in the B-ring side chain; the former has an asymmetry center on the B-ring -CH_2OH group with an αR, 6*S* configuration, while the latter contains a pi-bonded methylene group. The binding of the drugs to tubulin was measured by studying the intrinsic fluorescence of tubulin. Figure 2 shows the quenching of tubulin fluorescence by both drugs. As shown, both drugs quench the fluorescence of tubulin in a concentration dependent manner (Fig. 2). The quenching of fluorescence is abolished if the tubulin is denatured with urea (data not shown).

The affinity constants were measured by studying the fluorescence quenching at different drug concentrations. The data were analyzed by using either one-, two- or three-affinity Michaelis–Menten binding model as described under Experimental Procedures. The results (Fig. 3) show that the binding of THC 5 to tubulin followed a simple one-site binding model with a K_d value of 6 μM (A). In contrast, the binding of THC 18 did not follow a simple one-affinity model, rather, it followed a two-affinity model as judged by the analysis of residuals. In this case, the binding data showed two affinities: one high-affinity with a K_d value of 0.25 μM and a low affinity with a K_d value of 17 μM.

Binding of THC 18 to the tubulin isoforms $\alpha\beta_{II}$ and $\alpha\beta_{III}$. The two affinity values for the binding of THC 18 to PC-tubulin may be attributed either to the presence of different isoforms of tubulin or to the presence of different binding sites. In an effort to test these options we have studied the binding of this drug with pure β-tubulin isoforms $\alpha\beta_{II}$ and $\alpha\beta_{III}$. The results (Fig. 4) show that the drug binds to both the isoforms with one major affinity, although another minor species is exhibited. The minor species probably represents the presence of contaminating isoforms. However, in both cases, the amount of the minor species is negligible. For $\alpha\beta_{II}$ (top panel), 1.2 μM tubulin could be saturated with a drug concentration of 10 μM while the same for

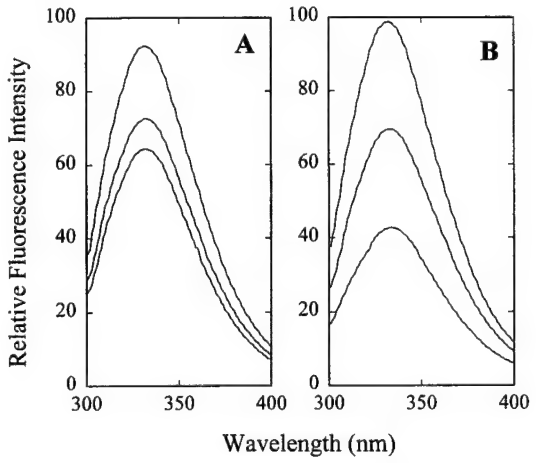

FIG. 2. Quenching of intrinsic tryptophan fluorescence of tubulin by the thiocolchicine analogs. PC-tubulin (1 μM) in Buffer A was incubated in the presence of different concentrations of THC 5 (A) and THC 18 (B) at 25°C for 30 min and the intrinsic tubulin fluorescence was measured at 300–400 nm upon excitation of the samples at 278 nm. Top curve, tubulin only; middle curve, tubulin + 2 μM drug; bottom curve, tubulin + 10 μM drug.

$\alpha\beta_{III}$ (Bottom panel) is above 60 μM. The K_d values for the major species are 0.5 μM and 17 μM for $\alpha\beta_{II}$ and $\alpha\beta_{III}$ respectively. Thus, the presence two affinity values for the binding of THC 18 to PC-tubulin are due to the presence of different tubulin isoforms, not due to the presence of two different binding sites.

The results are interesting in the sense that a minor difference in the B-ring side chain makes a significant difference with regard to the binding to different tubulin species. THC 5 with its -CH_2OH group cannot interact differentially with the tubulin isoforms, while THC 18 with its pi-bonded methylene group does it quite remarkably. It is worth mentioning that for allocolchicinoids with 7-membered B-ring and a 6-membered C-ring, similar change in the B-ring substituent did not change the antitumor property significantly (19).

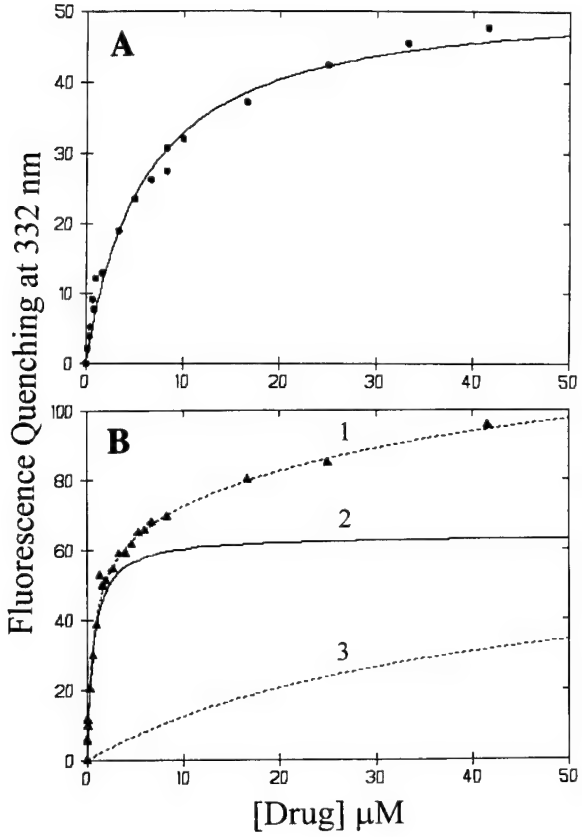

FIG. 3. Equilibrium binding of THC 5 and THC 18 with PC-tubulin. Aliquots of PC-tubulin (1.1 μM) in Buffer A were incubated with different concentrations of either THC 5 (A) or THC 18 (B) at 25°C for 1 h and the fluorescence was measured at 332 nm upon excitation of the samples at 278 nm. The fluorescence data were first corrected for the inner-filter effect and the amount of fluorescence quenching was measured by comparing with the fluorescence of the sample in the absence of drug. The quenching data at different drug concentrations were analyzed as described under Experimental Procedures. Notice that the data for THC 5 yielded only one affinity value of 7 μM, while those of THC 18 could be resolved into two components with the affinity values of 0.5 μM (upper dotted line) and 17 μM (lower dotted line).

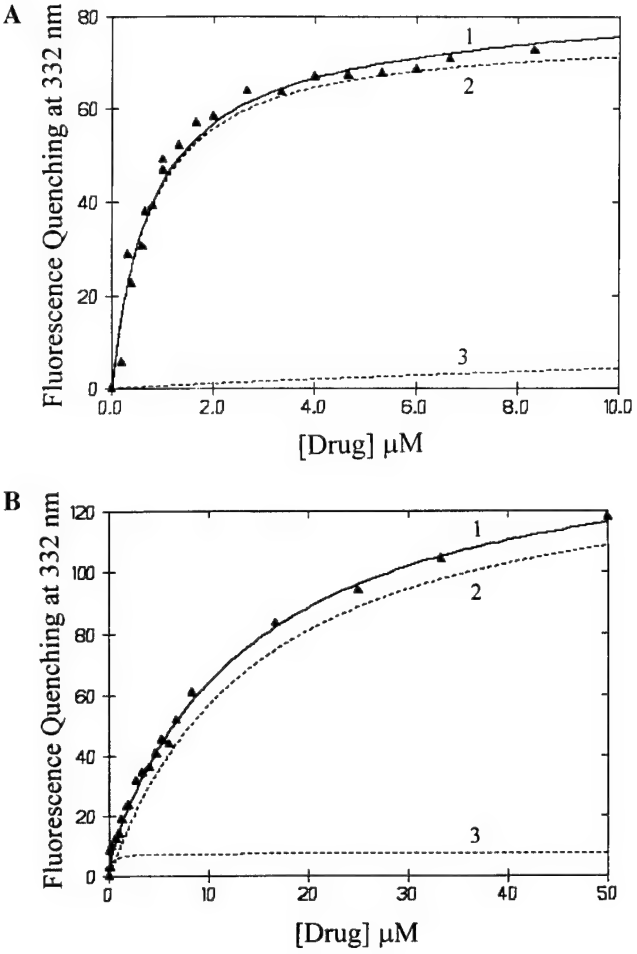

FIG. 4. Binding of THC 18 with the β-tubulin isoforms $\alpha\beta_{II}$ and $\alpha\beta_{III}$. Aliquots of β-tubulin isoforms (1.1 μM) in Buffer A were incubated with different concentrations of THC 18 at 25°C for 1 h. The intrinsic protein fluorescence of the samples were measured at 332 nm and the quenching of fluorescence at different drug concentrations were calculated as described for Fig. 3 (curve 1). The quenching data were analyzed by a two-affinity binding model and the curves for each affinity values were resolved. Curve 2, major component; curve 3, minor component. The minor components in each panel might represent contaminating isoforms of tubulin and are negligible. A, $\alpha\beta_{II}$; B, $\alpha\beta_{II}$.

Since the composition of tubulin isoforms changes from one tissue to the other, differential interaction of antitumor drugs with the isoforms will certainly affect the anti-tumor index of a drug for a certain tissue. Similar studies with other analogs of this series may yield valuable information regarding the rational design of tissue-specific antitumor drugs.

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A Monoclonal Antibody to α-Tubulin: Purification of Functionally Active α-Tubulin Isoforms

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A Monoclonal Antibody to α-Tubulin: Purification of Functionally Active α-Tubulin Isoforms†

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ABSTRACT: Both α- and β-tubulin exist as numerous isotypic forms that originate from different primary sequences as well as a variety of posttranslational modifications. Recent studies show that tubulin dimers differing in the β-subunit differ significantly in their subcellular distribution as well as in their functional properties such as assembly, dynamics, conformation, and interaction with antimitotic drugs; however, very little is known about the functional significance of the different α-tubulin isoforms and their posttranslational modifications. In an effort to get a better understanding about the α-tubulin isoforms, a monoclonal antibody, AYN.6D10, was prepared against the mammalian α-tubulin C-terminal sequence Glu-Glu-Gly-Glu-Glu-Tyr. Using an immunoaffinity column, bovine brain tubulin was fractionated into three functionally active αβ heterodimers which were identified by immunoblotting with α-tubulin-specific antibodies and sequence analysis. Assembly studies in the presence of glycerol and Mg^{2+} show that one of the fractions, that contains mainly the tyrosinated form of α1/2, assembled poorly, while the nontyrosinated form assembled normally. The results indicate that tubulin dimers differing in their α-tubulin may differ in their functional properties. Future studies with the isoforms may yield valuable information regarding the role of α-tubulin and its posttranslational modifications in regulating microtubule assembly and function in vivo.

Microtubules, the ubiquitous eukaryotic organelles, mediate various cellular functions such as cell division, motility and transport, maintenance of cell shape, and signal transduction (*1*, *2*). Tubulin, the major subunit of microtubules, is a heterodimeric protein consisting of two related peptides known as α- and β-tubulin (*1*, *2*). Both α- and β-tubulin exist as multiple isoforms which differ in their primary sequences as well as in the posttranslational modifications (*3*−*12*). In mammalian species, about six α-tubulin classes are encoded by the genes Mα1, Mα2, Mα3/7, Mα4, Mα6, and αTT1 (*3*−*12*) (the prefix "M" indicates that these genes were originally identified in the mouse). On the other hand, there are seven β-tubulin classes, designated as β_{I}, β_{II}, β_{III}, β_{IVa}, β_{IVb}, β_{V}, and β_{VI}, which have unique C-terminal sequences (*5*, *7*) [for reviews on tubulin isoforms, see (3), (7), and (9)]. The polypeptides α1 and α2 differ only at amino acid 232 (Gly/Ser) and are expressed mainly in the brain and also to some extent in other tissues; α3/7 is a form that is encoded by two different genes, α3 and α7, which are expressed only in the testes; α4 is unique in the sense that it lacks the coded C-terminal tyrosine residue, and is expressed constitutively in many tissues but mainly in the brain, muscle, and heart; αTT1 is found only in the testes (*6*); α6 is expressed in the liver and stomach. Since α1 and α2 differ in just one amino acid, they will be grouped here as α1/2. Of these five different α-tubulin classes, α1/2 and α4 are the α-tubulins found in the brain (*5*). It is known that α1/2 and α4 both occur in birds (*3*, *7*, *9*), suggesting that the differences among them have been conserved at least since the divergence of the mammalian and avian lines 310 million years ago. The strong conservation of these differences suggests that they are functionally significant. Although the α-tubulin classes do not have unique C-terminal sequences as do the β-tubulin classes, significant sequence differences are found among different α-tubulins. The C-terminal sequences for different α-tubulin classes are depicted in Figure 1.

Tubulin undergoes posttranslational modifications that include (a) tyrosination and detyrosination of α-tubulin (*13*, *14*); (b) acetylation of α-tubulin at Lys^{40} (*15*, *16*); (c) loss of both the C-terminal tyrosine and the penultimate glutamic acid residue from α-tubulin, resulting in the formation of Δ2 tubulin[1] (*17*); (d) polyglutamylation of both α- and β-tubulin, in which glutamic acid residues are added laterally through a γ-carboxyl of a glutamic acid at the C-terminal region through an isopeptide linkage (*18*−*20*); (e) polyglycylation of both α- and β-tubulin (*21*); and (f) phosphorylation of the β_{III} isoform (*22*−*24*).

To understand the roles each tubulin isoform can play in vivo, it would be necessary to purify an isoform in the functionally active form. Thus, we have generated monoclonal antibodies specific for the β_{II}, β_{III}, and β_{IV} isoforms using the C-terminal peptides corresponding to each isoform (*25*−*27*). Using these antibodies on immunoaffinity columns, the β-tubulin isoforms $\alpha\beta_{II}$, $\alpha\beta_{III}$, and $\alpha\beta_{IV}$ have been

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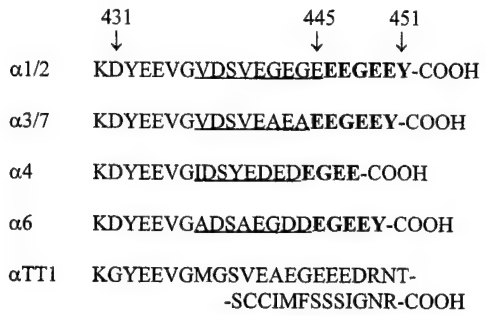

FIGURE 1: C-terminal sequences for the mammalian α-tubulin classes. The sequences from residue 430 through the C-termini are shown. The glutamic acid residue at 445 (indicated by an arrow) is the residue that is the site of posttranslational polyglutamylation. The sequence differences among different classes are underlined. The antigenic sequence used for making the antibody AYN.6D10 is in boldface letters.

purified in functionally active forms (*27*). The studies with purified β-tubulin isoforms reveal that they differ significantly in drug binding (*28*−*30*), drug-induced conformational changes (*31*), assembly (*27*), and in the dynamics of the microtubules they form (*32*). Although ample evidence has accumulated to demonstrate that the β-tubulin isoforms indeed differ in their functional properties, no such information is available on α-tubulin. One major reason may be that it has not been possible to make a monoclonal antibody specific for any α-tubulin class. Thus, this project was initiated to generate monoclonal antibodies specific for different α-tubulin classes. I have generated a monoclonal antibody by using a synthetic peptide corresponding to the C-terminal hexapeptide (EEGEEY) of most α-tubulins (Figure 1). By using the immunoaffinity column, bovine brain tubulin was fractionated into four fractions according to the nature of their α-subunit. In vitro assembly studies were carried out to study the functional differences among different α-tubulin fractions.

EXPERIMENTAL PROCEDURES

Materials. The C-terminal peptide for the α-tubulin isoform NH_2-Cys-Glu-Glu-Gly-Glu-Glu-Tyr-COOH was custom-synthesized by Genosys Biotechnologies, Inc., Woodlands, TX. The cysteine residue at the NH_2-terminal is required for coupling the peptide with the carrier protein. Freund's adjuvants, maleimide-activated KLH, or BSA was obtained from Pierce. Poly(ethylene glycol) (PEG 1500) was obtained from Boehringer-Mannheim. Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). Carboxypeptidase A and the mouse monoclonal antibody to tyrosinated tubulin (clone TUB.1A2) were obtained from Sigma. The polyclonal antibody to Glu tubulin was a kind gift from Drs. J. C. Bulinski and G. G. Gunderson, Columbia University, New York, NY. HRP-labeled antibodies were obtained from Pierce. The chemiluminescent substrate (Enhanced NuGlow) for immunoblot development was obtained from Alpha Diagnostic Intl. Inc., San Antonio, TX.

Coupling of the Antigen with the Carrier Protein. The peptide was coupled to KLH or BSA according to the instructions provided by the manufacturer. Three milligrams of the peptide dissolved in 40 μL of dimethyl sulfoxide was incubated with 300 μL of maleimide-activated BSA or KLH (10 mg/mL) at the room temperature for 2 h. The mixture was diluted to 3 mL and then dialyzed for 14 h against sterile PBS using sterile dialysis tubing. The peptide was stored in aliquots at −20 °C.

Immunization of Mice and Preparation of the Monoclonal Antibody. Two female balb/c mice (4 weeks old) were injected initially with the KLH-peptide (approximately 100 μg) in Freund's complete adjuvant. After 2 weeks, the mice received a second injection with Freund's incomplete adjuvant. After 10 days, the mice were bled through the tail, and the sera were checked for antibody titer in an ELISA using bovine brain PC−tubulin. None of the mice exhibited positive. After 10 days, the mice were injected with peptide−BSA in the incomplete adjuvant. After 10 days, the mice were bled, and the antibody titer was checked. Both mice exhibited significant titer even at a serum dilution of 1:5000. After 10 days, one of the mice was injected intraperitoneally with the peptide−BSA without adjuvant. After 3 days, the mouse was sacrificed, and the spleen was removed. The spleen cells were fused with SP2/0 myeloma cells in the presence of 50% PEG 1500. After the fusion, the cells were diluted in 200 mL of HAT selection medium containing 20% fetal bovine serum, and were subsequently plated in 20, 96-well culture plates and kept in a humidified CO_2 incubator for 1 week. The colonies started appearing after 1 week. The colonies were checked for antibody titer in ELISA assay with PC−tubulin. Out of 1920 fusion wells, only 278 had colonies in them while only 7 showed positive against brain tubulin. These seven clones were single-cell-cloned on gamma-ray-irradiated rat thymocytes. Out of these seven antibodies, four were of IgM type, one was IgG_1, and two were IgG_{2b}. One clone (AYN.6D10) was grown up in roller bottles, and the monoclonal antibody was purified on a protein A−agarose column. Isotyping shows that the antibody is an IgG_{2b} having both light chains of the κ-type.

Preparation of the E. coli Fusion Proteins Containing the C-Terminal Sequence of Each α-Tubulin Class. The fusion proteins (kind gifts from Drs. Nicholas J. Cowan and Sally Lewis, New York University Medical Center, New York, NY) were made by fusing the *E. coli* tryptophan synthetase gene (trp E) with the cDNA sequence corresponding to the C-terminal portion of each α-tubulin (*33*). For the α-tubulin classes α1/2, α3/7, α3/7 −Y, and α6, the C-terminal portion included residue 254 to the C-terminal end; for α4 and α4 +Y, the portion included residue 168 to the C-terminal end. Thus, the fusion proteins for α4 and α4 +Y are larger in size as compared to the others. The C-terminal tyrosine residue was added to α4 by site-directed mutagenesis of a termination codon to one that codes for a tyrosine residue.

[1] Abbreviations: α3/7 −Y, α3/7 tubulin lacking the tyrosine residue at the C-terminus; α4 +Y, α4 tubulin with an additional tyrosine residue at the C-terminus; BSA, bovine serum albumin; buffer A, 0.1 M MES-Na, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 1 mM GTP; EDTA, ethylenediaminetetraacetate; Δ2 tubulin, α-tubulin lacking the C-terminal tyrosine and glutamic acid residues; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; ELISA, enzyme-linked immunosorbent assay; Glu tubulin, α-tubulin lacking the C-terminal tyrosine residue; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC-tubulin, tubulin purified by phosphocellulose chromatography; PBSTT, phosphate-buffered saline containing 0.02% thimerosal and 0.1% Tween-20; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tyr tubulin, α-tubulin having a tyrosine residue at the C-terminus.

Similarly, the C-terminal tyrosine residue was removed from the α3/7 sequence to form the α3/7 −Y sequence.

Preparation of Tubulin. Microtubules were purified from bovine brain cortex by a cycle of assembly and disassembly, and tubulin was purified from microtubules by phosphocellulose chromatography as described elsewhere (*34*). All purifications were carried out in buffer A that contains 0.1 M MES-Na, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 1 mM GTP.

Preparation of α-Tubulin Fractions for Functional Studies. Tubulin fractions obtained from the immunoaffinity column were identified by a Bradford protein assay, pooled separately, and concentrated on Amicon ultrafiltration unit. The fractions were dialyzed against buffer A to remove NaCl, were concentrated by overnight (14 h) dialysis in buffer A containing 8 M glycerol, and were stored frozen in aliquots at −80 °C in buffer A (0.1 M MES-Na, pH 6.4, 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, and 1 mM GTP) containing 8 M glycerol. Glycerol was removed before each experiment by repeated dilution and centrifugation using Centricon 30 membrane filtration units.

Preparation of the Immunoaffinity Column. The antibody AYN.6D10 was coupled to CNBr-activated Sepharose according to the instructions provided by Pharmacia for CNBr-activated Sepharose 4B as described in Banerjee et al. (*25*). About 63 mg of antibody was incubated with 10 g of CNBr−Sepharose 4B at 4 °C for 12−16 h with gentle shaking. The unoccupied sites were blocked by overnight incubation with 1.0 M ethanolamine hydrochloride (pH 9.0). The resin was subsequently subjected to three cycles of alternating pH washes (sodium acetate buffer, pH 4.0, and Tris-HCl buffer, pH 8.0). The resin was finally resuspended in 0.1 M MES buffer (pH 6.4) and was stored with 0.02% sodium azide.

Gel Electrophoresis and Immunoblotting. Gel electrophoresis was carried out in polyacrylamide gels (10 cm long) in the presence of sodium dodecyl sulfate according to Laemmli (*35*). Tubulin samples were reduced and carboxymethylated (*36*) prior to the run. Protein bands from the gels were transferred onto a nitrocellulose membrane using a BIO-RAD Trans-Blot cell as described elsewhere (*25*). For immunoblotting, the nitrocellulose blots were blocked in 5% Carnation nonfat milk and 0.1% BSA in PBSTT buffer for 2 h, incubated with the primary antibody for 1 h, and washed 3 times with PBSTT with mild shaking. The blots were incubated with HRP−antibody for 1 h followed by 3 washes with PBSTT. The blots were developed using chemiluminescent substrate and were exposed immediately for 5 min on KODAK X-Omat AR film.

Preparation of Mouse Polyclonal Antiserum to α4 Tubulin. Mouse polyclonal antiserum to α4 tubulin was prepared by repeatedly immunizing 3−4 week old female balb/c mice with a synthetic peptide, Ile-Asp-Ser-Tyr-Glu-Asp-Glu, corresponding to the α4 tubulin sequence (amino acid residues 437−443). Each mouse was injected subcutaneously with about 100 μg of the BSA-conjugated peptide in PBS buffer along with the Freund's adjuvants. After four immunizations, the mice were bled through their tails and the sera were collected.

Preparation of α-Tubulin for Sequencing Studies. The details of the steps leading to sequence analysis are outlined in Figure 7. The protein samples (without carboxymethylation) were boiled for 5 min with an equal volume of 2× Laemmli sample buffer and were run on preparative SDS gels (using SDS from Sigma)[2] (6%, 5 mm thick) to separate α- and β-tubulin. About 500 μg of tubulin samples was run at a time. After the electrophoresis, the tubulin bands were visualized either by staining and destaining or by immersing the gel in ice-cold 4 M sodium acetate. The protein bands were cut out and were taken in glass tubes (10 × 1 cm), and the protein was electro-eluted according to Rushbrook (*37*) using a vertical tube gel apparatus. Elution was carried out at room temperature for 14 h at a constant current of 10 mA per tube using Laemmli running buffer, and the eluted protein was collected in dialysis tubing. The protein was precipitated with 10 volumes of superchilled acidified acetone (acetone:1 N HCl, 40:1, v/v) to remove the SDS, dissolved in 0.1 M Tris HCl (pH 9.0). This cycle was repeated twice, and finally the protein was dialyzed in the above buffer for sequencing.

Sequence Analysis. Protein samples in 0.1 M Tris-HCl (pH 9.2) were digested with endoproteinase Lys-C (17 μg/mL) at room temperature for 14 h. The enzyme cleaves at the C-terminal peptide bond of each lysine residue. For α-tubulin, the cleavage at the lysine residue (Lys^{430}) is readily achieved, leaving 18−21 amino acid long C-terminal peptides (*38*). Fifteen micrograms of the tubulin digest was subjected to reversed-phase HPLC on an Applied Biosystems ABI 130 HPLC using a Phenomenex Atlantis C18 column (50 × 2 mm). Solvent A was 0.1% TFA in water; solvent B was 0.085% TFA in 70% acetonitrile in water. The chromatography was performed using a gradient of 0−60% solvent B in 60 min at a flow rate of 0.5 mL/min. The peaks containing the C-terminal fragments were identified by sequence analysis. Sequencing was performed on an Applied Biosystems 477 A protein sequencer by the automated Edman degradation method using aryl-covalent membrane (Millipore). PTH-amino acids were separated using ABI 120 HPLC.

Other Methods. Microtubule assembly and enzyme-linked immunoassays (ELISA) were performed as described in Banerjee et al. (*25*). Protein measurements were done according to Lowry et al. (*39*). For column-eluted fractions, protein was measured according to the dye-binding method of Bradford (*40*).

RESULTS

Characterization of the Monoclonal Antibody AYN.6D10 by Immunoblot Analysis. The antibody was tested against bacterial fusion proteins corresponding to the C-terminal sequences of different α-tubulin classes by SDS−PAGE and immunoblotting. As shown in Figure 2, the antibody recognized α1/2, α3/7, α6, and α4 +Y. The α-tubulin classes that were not recognized by the antibody are Mα4 and Mα3/7 −Y, both of which lack the C-terminal tyrosine. In an attempt to study whether the C-terminal tyrosine is the epitope for the antibody, a detyrosination experiment was performed. For this, the fusion proteins were subjected to SDS−PAGE as before, and the gel was transferred onto a nitrocellulose membrane. The blot was subsequently treated with carboxypeptidase A (4 μg/mL, 2 h) to remove the C-terminal tyrosine from the fusion proteins and was

[2] For unknown reason, the SDS purchased from Sigma Chemical Co. gives the best separation of α- and β-tubulin bands for samples which were not reduced and carboxymethylated.

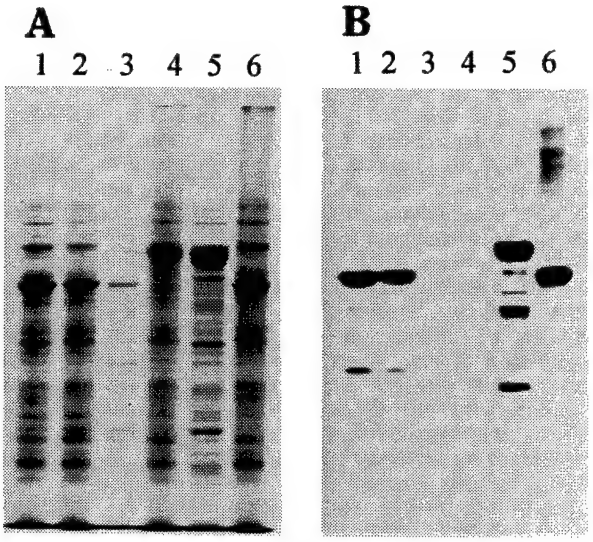

FIGURE 2: Specificity of monoclonal antibody AYN.6D10. *E. coli* extracts containing trp E fusion proteins corresponding to the C-terminal portion of Mα1/2 (lane 1), Mα3/7 (lane 2), Mα3/7 −Y (lane 3), Mα4 (lane 4), Mα4 +Y (lane 5), and Mα6 (lane 6) were subjected to SDS−PAGE (8%). An identical gel was transferred onto a nitrocellulose membrane and was processed for immunoblotting using AYN.6D10 as the primary antibody (1:10 000 dilution), HRP-labeled secondary antibody, and an enhanced chemiluminescent substrate. Panel A: Coomassie blue stained gel. Panel B: blot. Notice that the antibody did not react with the fusion proteins that lacked a C-terminal Tyr residue. Minor bands in the blot are due to the degradation of fusion proteins in the bacterial extract, which is almost unavoidable.

subsequently tested by immunoblotting. The results showed that none of the lanes were recognized by the antibody (data not shown). When the same blot was treated with the anti-Glu tubulin antibody, all the lanes were lighted up. Thus, the results of the immunoblot analysis showed that the monoclonal antibody AYN.6D10 recognizes all the α-tubulin classes with a C-terminal tyrosine residue and that the antibody recognition is abolished upon removal of the C-terminal tyrosine by digestion with carboxypeptidase A.

Chromatography of Bovine Brain Tubulin on the Antibody−Sepharose Column. In an effort to test the tubulin-binding property of the immunoaffinity column, PC−tubulin was fractionated on the column as outlined in Figure 3. After the elution of the unbound fraction (fraction A), the column was washed well with the buffer. The column was then eluted with a linear salt gradient of 0−1 M NaCl. As shown in Figure 4, the bound protein was eluted as two well-separated peaks, fractions B (peak I) and C (peak II). After this gradient elution, the column was finally eluted with 3 M KI to get the remaining bound protein (fraction D). The relative yields for the fractions were calculated from the area under the peaks from Figure 4. Thus, the relative amounts are found to be 50.2%, 29.4%, 14.1%, and 6.3% for fractions A, B, C, and D, respectively. To test the functional activity, the tubulin fractions (except the KI-eluted fraction D) were dialyzed to remove the salt, were concentrated by overnight dialysis in buffer A containing 8 M glycerol, and were stored in aliquots at −80 °C.

Determination of α4 Content in the Isoform Fractions. Since bovine brain α-tubulin contains only α1/2 and α4 isoforms, it was necessary to see whether different α-tubulin isoform fractions differ in the composition of α1/2 and α4.

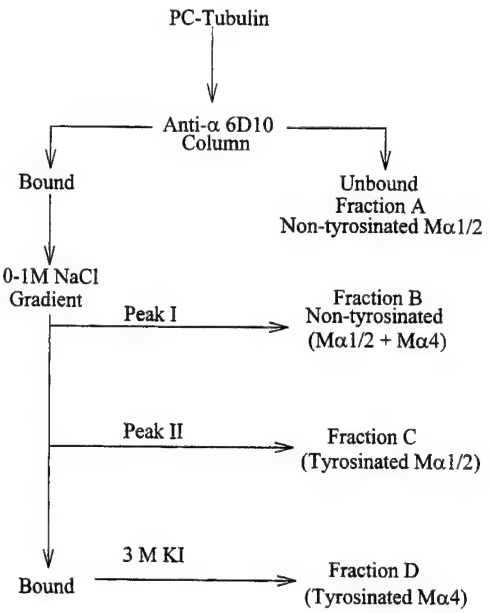

FIGURE 3: Schematic outline for the purification of the α-tubulin isoforms.

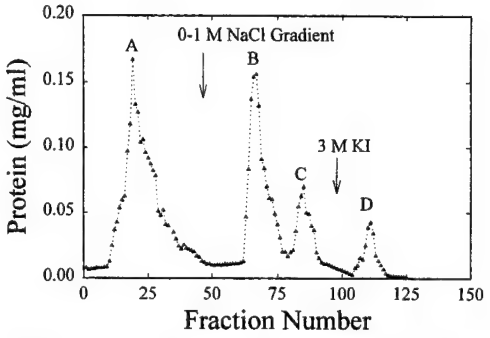

FIGURE 4: Fractionation of bovine brain tubulin on an immunoaffinity column containing the monoclonal antibody AYN.6D10. Bovine brain PC−tubulin (7 mg) was passed through the antibody−Sepharose column (15 mL, containing 30 mg of antibody) equilibrated in buffer A, and the unbound fraction was collected (each fraction was 1.5 mL). The column was subsequently washed with 50 mL of the same buffer, and the bound fraction was first eluted with a linear salt gradient of 0−1 M NaCl. Protein was determined in all the fractions by a Bradford protein assay. Notice that the bound fraction eluted as two peaks (B and C). The two peaks were pooled separately. After the elution of B and C, the remaining bound fraction was eluted with 3 M KI in buffer A.

A polyclonal antiserum against α4 was used to test the presence of α4 in those fractions. The presence of α4 was checked by SDS−PAGE and subsequent immunoblotting analysis with mouse polyclonal anti-α4 antiserum. The result (Figure 5) shows that the NaCl-eluted fraction B and the KI-eluted fraction D light up with the anti-α4 antiserum, while the unbound fraction A and the NaCl-eluted fraction C do not. These data clearly indicate that fractions A and C are completely depleted of α4 and thus contain mainly α1/2.

Determination of the Posttranslational Modifications in α-Tubulin in the Isoforms. Since the antibody AYN.6D10 was found to react only with the tyrosinated forms of the α-tubulin classes, it was interesting to see whether the four fractions obtained by immunoaffinity column chromatography differ in their tyrosination states. To determine the tyrosination state of tubulin, immunoblot analysis was

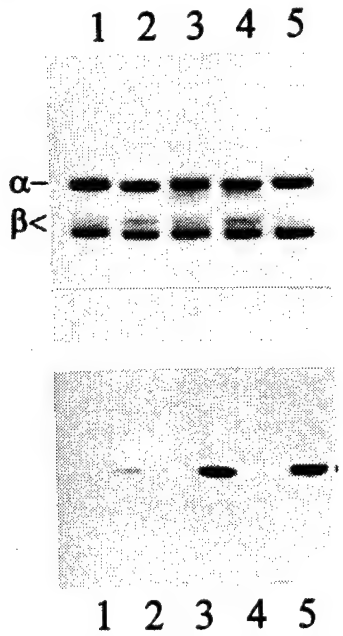

FIGURE 5: Immunoblot analysis of the tubulin isoforms with anti-α4 antiserum. PC−tubulin was passed through the immunoaffinity column as described for Figure 3. After collection of the unbound fraction and subsequent NaCl elution, the remaining bound fraction was eluted with 3 M KI. All the fractions were subsequently reduced and carboxymethylated. The samples were subsequently analyzed by SDS−PAGE (6%) and immunoblotting with the anti-Mα4 antiserum using an enhanced chemiluminescent substrate. Upper panel, Coomassie-stained gel; lower panel, immunoblot. Samples in each lane are as follows: lane 1, PC−tubulin; lane 2, unbound (fraction A); lane 3, fraction B; lane 4, fraction C; and lane 5, fraction D. 2.5 μg of each sample was loaded in each lane. Note that the anti-α4 antibody recognizes lanes 1, 3, and 5 only, which indicates that Mα4 tubulin is absent in fractions A and C.

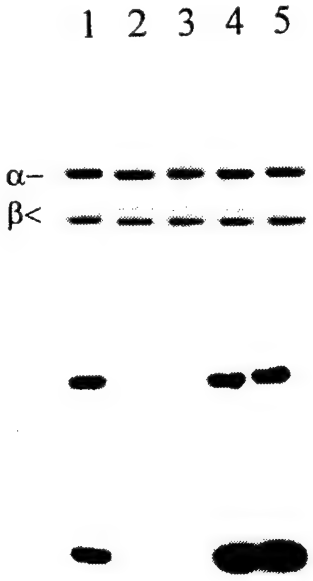

FIGURE 6: SDS−PAGE and immunoblot analysis of the tubulin fractions obtained by immunoaffinity chromatography on the antibody column. Reduced and carboxymethylated tubulin samples were analyzed by SDS−PAGE (6%) and immunoblotting with antibodies to tyrosinated and nontyrosinated tubulin. Top panel, Coomassie blue stained gel; middle and bottom panels, immunoblots developed with an enhanced chemiluminescent substrate. The primary antibodies are middle panel, AYN.6D10; bottom panel, TUB.1A2 (antityrosinated tubulin). The samples in each lane are as follows: lane 1, PC−tubulin; lane 2, unbound fraction A; lane 3, NaCl-eluted fraction B; lane 4, NaCl-eluted fraction C; lane 5, KI-eluted fraction D. 2.5 μg of each fraction was loaded in each lane.

performed with the monoclonal antibody AYN.6D10 and also with the antibody to tyrosinated tubulin. The results (Figure 6) show that the antibody AYN.6D10 recognized the α-tubulin bands in unfractionated tubulin, and in fractions C and D. No staining was visible for the α-tubulin bands in fractions A and B. None of the β-tubulin bands were recognized by the antibody. These results indicate that the α-tubulin present in the unbound fraction A and in the NaCl-eluted fraction B is not tyrosinated. Similar results were also obtained when the immunoblotting was done with the monoclonal antibody TUB.1A2 (Sigma) which is specific for tyrosinated tubulin (Figure 6). It should be mentioned here that, although the antibody is specific for tyrosinated tubulin, fraction B that contains the detyrosinated tubulin can still bind to the immunoaffinity column. This is probably due to the nonspecific interaction caused by a very high concentration of antibody in the column (above 3 mg/mL). This will be discussed later under Discussion.

Sequencing Studies. To identify the α-tubulin classes in the above fractions, sequence analysis was performed. The details of the procedures leading to sequence analysis are outlined in Figure 7. The α-tubulin bands were cut out from SDS gels and were isolated by electro-elution. The α-tubulin isolated from the above fractions was digested with endoproteinase Lys-C (which cleaves at the C-terminal of each lysine residue), and the fragments were separated by reversed-phase HPLC on a C-18 column. All the fractions were partially sequenced (up to five residues from the NH_2-terminal) to identify the C-terminal fragment, which was sequenced

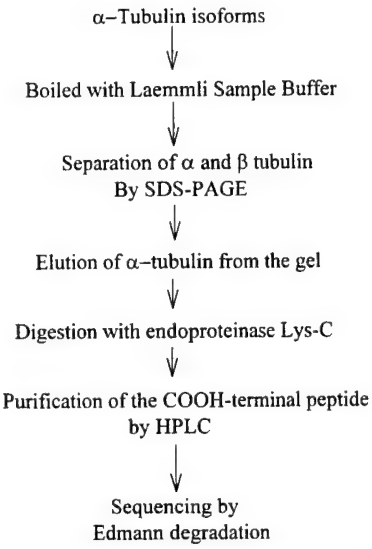

FIGURE 7: Schematic outline for the analysis of different tubulin fractions.

completely. As shown in Figure 8, the C-terminal peptides were eluted at 30−35 min. The unbound fraction (fraction A) showed the two peaks 'a' and 'b' which eluted at around 30−32 min (Figure 8, panel A). Edman degradation of peak 'a' gave the sequence DYEEVGVDSVEGEGEEEGEE, which matches exactly with the C-terminal sequence of α1/2, while peak 'b' gave the sequence DVNAAIATIK ($\alpha^{327-336}$), which is also specific for α1/2. It should be mentioned here

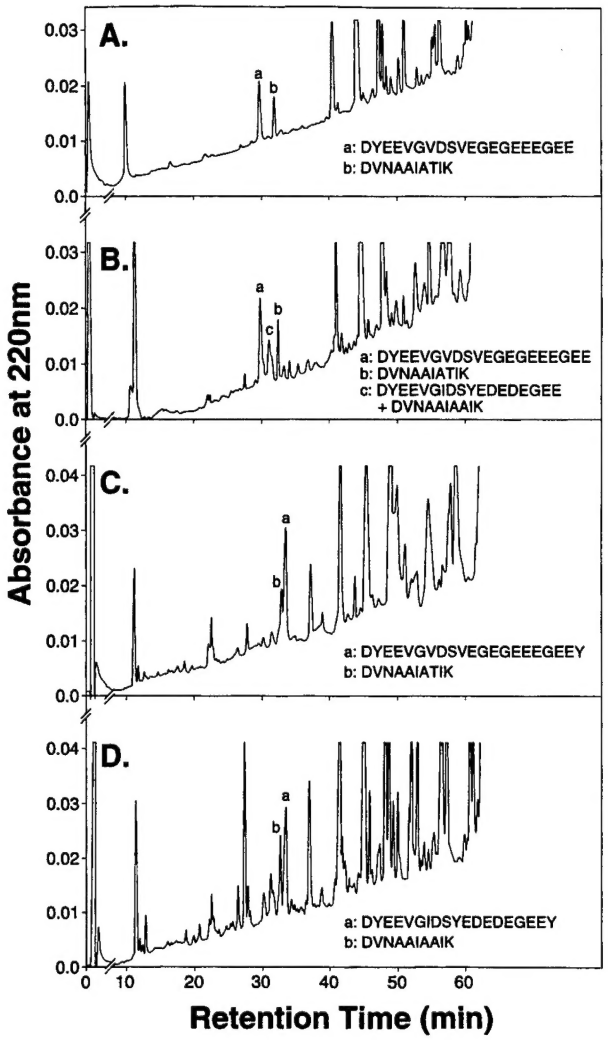

FIGURE 8: HPLC separation of α-tubulin peptides obtained by digesting tubulin fractions with endoproteinase Lys-C. Four tubulin fractions, A, B, C, and D, obtained by fractionating bovine brain tubulin on an AYN.6D10 immunoaffinity column, were subjected to SDS−PAGE on preparative gels, and the α-tubulin bands were cut out and electro-eluted as described under Experimental Procedures. Isolated α-tubulin samples (10 μg) were digested with endoproteinase Lys-C (17 μg/mL) in 0.1 M Tris-HCl (pH 9.2) for 14 h. The tubulin digests were subjected to HPLC using a C-18 column. The C-terminal peptides were identified by sequence analysis using the automated Edman degradation method. Panel A, unbound fraction (fraction A); panel B, NaCl-eluted peak I (fraction B); panel C, NaCl-eluted peak II (fraction C); panel D, KI-eluted fraction (fraction D). The sequences are shown for peptides that are labeled with the letters 'a', 'b', and 'c' in each panel.

that the signals for the amino acid residues Glu^{445}, Glu^{449}, and Glu^{449} (underlined residues in the above sequence) were significantly low. This will be discussed later. Thus, the unbound fraction seems to be a mixture of the nontyrosinated forms (that may also include the Δ2 form) of α1/2 (the term "non-tyrosinated" is designated for its reactivity with anti-tyrosinated tubulin antibody). All the other peaks were also partially sequenced, and no other peak was found to contain the sequence corresponding to the C-terminal peptide.

In the case of fraction B, three peaks, 'a', 'b', and 'c', were obtained in this region (Figure 8B). The positions of peaks 'a' and 'b' were identical to those of peaks 'a' and 'b' in fraction A. In this case, a new peak, peak 'c', eluted between peaks 'a' and 'b'. Sequence analysis showed that peak 'a' is DYEEVGVDSVEGEGEEEGEE, which matches the C-terminal sequence of α1/2, and peak 'b' was found to be DVNAAIATIK, which is also specific for α1/2. Peak 'c', which was not found in the unbound fraction A, was found to be a mixture of two peptides: DYEEVGIDSYEDEDEGEE and DVNAAIAAIK; the former matches the C-terminal sequence of α4, and the latter matches the α4 sequence ($\alpha^{327-336}$) (α1/2 differs from α4 in the amino acid residue at 333, which is a threonine in α1/2 and an alanine in α4). In this context, it should be mentioned that in the sequencing chromatogram signals for 2 amino acid residues were obtained for each of the first 10 cycles. The amino acid residues obtained for the first 10 cycles are: D, Y/V, E/N, E/A, V/A, G/I, I/A, D/A, S/I, Y/K. Only a single sequence was obtained for each of cycles 11−20. The amino acid sequence obtained from the 11th through the 18th cycle is EDEDEGEE, which is the C-terminal sequence of α4. No other combination matched with any known tubulin sequence. Thus, fraction B seems to be a mixture of the nontyrosinated forms of α1/2 and α4. In this case also, the sequencing chromatogram exhibited significantly low signals for the underlined residues.

In the case of the NaCl-eluted fraction C, the two closely positioned peaks 'a' and 'b' were obtained (Figure 8C). In this case, peak 'a' eluted slightly more slowly than peak 'a' in fractions A and B. This peak 'a' was found to have the sequence DYEEVGVDSVEGEGEEEGEEY while peak 'b' had the sequence DVNAAIATIK, which are both specific for α1/2. No sequence corresponding to α4 tubulin was detected by Edman degradation. Thus, fraction C is essentially the tyrosinated form of α1/2. Again, like the other fractions, the sequencing chromatogram exhibited significantly lower signals for the underlined residues.

In the case of the KI-eluted fraction (fraction D), four peaks were obtained. In addition to the two peaks 'a' and 'b' (Figure 8D), other minor peaks were observed corresponding to peaks 'a', 'b', and 'c' in fractions A and B. Two major peaks, 'a' and 'b', were sequenced. Peak 'a' gave the sequence DYEEVGIDSYEDEDEGEEY, which corresponds to the C-terminal sequence of the tyrosinated form of α4 tubulin. Peak 'b' gave the sequence DVNAAIAAIK, which also matches the α4 sequence ($\alpha^{327-336}$). Thus, this fraction is predominantly the tyrosinated form of α4, although it contains minor amounts of fragments which appear to correspond to the nontyrosinated forms of α1/2 and α4.

Colchicine-Binding Activity of the Tubulin Fractions. One of the major functional properties of tubulin is to bind the antimitotic alkaloid colchicine (*1*, *2*). The binding is slow but stable, and is associated with an increase in drug fluorescence which can be used to monitor the binding (*41*). The ability of tubulin to bind colchicine is very sensitive to the native structure of tubulin, and the binding activity decays with a half-life of about 4−6 h at 37 °C (*2*, *41*). To test whether fractions A, B, and C retain their native structures, the colchicine-binding pattern was studied. For this, the tubulin fractions (2 μM) were incubated with colchicine (100 μM) at 37 °C for 1 h, and the increase in fluorescence emission was recorded at 437 nm upon excitation of the samples at 380 nm. The amount of bound colchicine was calculated from the fluorescence values for the 1 μM tubulin−colchicine complex as described before (*28*). The

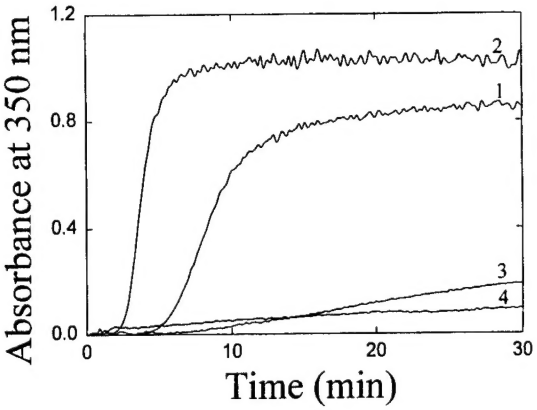

FIGURE 9: Assembly of α-tubulin isoforms in the presence of glycerol and magnesium. Aliquots of tubulin fractions (1 mg/mL) in buffer A containing 16 mM $MgCl_2$ and 4 M glycerol were incubated at 37 °C, and the assembly was studied by monitoring the turbidity of the samples at 350 nm. Different curves are as follows: curve 1, PC−tubulin; curve 2, fraction A; curve 3, fraction B; curve 4, fraction C.

Table 1: Functional Properties of the Different Tubulin Fractions Eluting from the AYN.6D10 Immuonoaffinity Column

tubulin fractions	elution from column	tyrosination status	α-tubulin class	colchicine binding	assembly
fraction A	unbound	nontyrosinated	α1/2	normal	normal
fraction B	NaCl peak I	nontyrosinated	α1/2, α4	normal	poor
fraction C	NaCl peak II	tyrosinated	α1/2	normal	poor
fraction D	3 M KI	tyrosinated	α4	not tested	not tested

amounts of bound colchicine were 0.82 ± 0.12, 0.8 ± 0.15, 0.85 ± 0.11, and 0.88 ± 0.12 (moles of colchicine per mole of tubulin dimer) for PC−tubulin, fraction A, fraction B, and fraction C, respectively. The data indicate that the tubulin fractions A, B, and C are functionally active with regard to their colchicine-binding activity.

Assembly of α-Tubulin Isoforms in the Presence of Mg^{2+}. PC−tubulin is known to assemble in the presence of high Mg^{2+} and glycerol to form polymers resembling normal microtubules (*42*). The isoforms were tested for their assembly activity at a single tubulin concentration in the presence of Mg^{2+} and glycerol. As shown in the figure, the rate and the extent of assembly for the unbound fraction A are little higher than those of the unfractionated PC−tubulin (Figure 9). This small but reproducible increase in assembly for fraction A has always been observed. On the other hand, fractions B and C assembled poorly. The extent of assembly for fractions B and C is found to be about 5−10% as compared to that of unfractionated tubulin. The details of the characterization of different tubulin fractions including their functional properties are summarized in Table 1.

Copolymerization of Fraction C with the Unbound Fraction. In an effort to study whether the tubulin from fraction C is capable of assembly, a copolymerization experiment was performed. Here, the unbound fraction was incubated with increasing concentrations of fraction C in the presence of glycerol and magnesium for 30 min, and the microtubules were pelleted on a glycerol cushion at 120000*g* for 5 min in an airfuge. The assembled microtubules were resuspended, boiled with Laemmli sample buffer, and analyzed by SDS−PAGE followed by immunoblotting with the monoclonal antibody AYN.6D10. By virtue of the fact that the antibody recognizes fraction C but not the unbound fraction A, it was

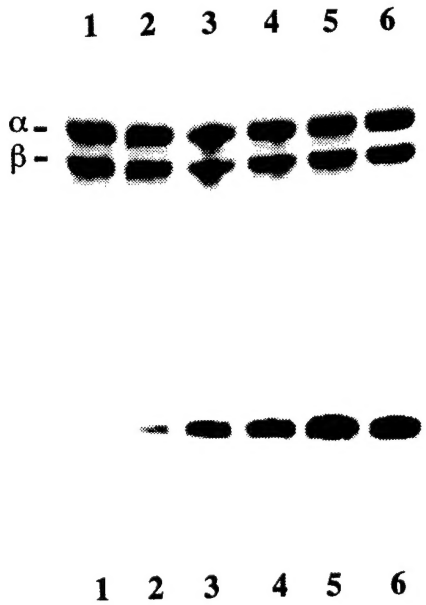

FIGURE 10: Coassembly of fraction C along with fraction A. Aliquots of fraction A (1 mg/mL) in buffer A containing 16 mM $MgCl_2$ and 4 M glycerol were incubated in the presence of different concentrations (0−0.25 mg/mL) of fraction C at 37 °C for 30 min. The microtubules were pelleted by centrifugation on a 50% sucrose cushion in an airfuge. The pellets were resuspended in Laemmli sample buffer and boiled for 5 min before being subjected to SDS−PAGE on 6.5% polyacrylamide gels (upper panel). An identical gel was processed for immunoblotting with the monoclonal antibody AYN.6D10 (lower panel). Since the antibody recognizes fraction C only and not fraction A, the blot demonstrates the ability of fraction C to copolymerize with fraction A. The amount of tubulin loaded in each lane is 5 μg. Fraction C concentrations are: lane 1, 0; lane 2, 0.05 mg/mL; lane 3, 0.10 mg/mL; lane 4, 0.15 mg/mL; lane 5, 0.20 mg/mL; and lane 6, 0.25 mg/mL.

easy to detect the amount of fraction C copolymerized in the pellets. The results show that the amounts of fraction C coassembled with unbound fraction A increased in a concentration-dependent manner (Figure 10). This indicates that although fraction C is unable to assemble by itself, it can copolymerize along with fraction A. That the pelleting of peak C protein was not due to a concentration-dependent aggregation of tubulin in the pellet was confirmed in a control experiment, in which fraction C (at the highest concentration used for the coassembly experiment) was incubated in an identical manner in the absence of fraction A and was centrifuged. No pellet was obtained, indicating that the coassembly of fraction C is not likely due to the concentration-dependent aggregation of tubulin.

DISCUSSION

The results of the immunoblot analysis of different α-tubulin fusion proteins show that the monoclonal antibody AYN.6D10 recognizes most of the mammalian α-tubulin classes containing a C-terminal tyrosine residue and that antibody recognition is lost upon removal of the C-terminal tyrosine by digestion with carboxypeptidase A. The antibody showed no reactivity with β-tubulin. Thus, the antibody appears to be another anti-tyrosinated tubulin antibody.

Immunoaffinity fractionation of bovine brain tubulin on the AYN.6D10 column yielded four tubulin fractions designated A, B, C, and D, where A is the unbound fraction, B and C are the fractions that eluted with a linear salt gradient,

and D is the fraction that was eluted with 3 M KI after the elution of B and C. Immunoblot analysis of the fractions with mouse anti-Mα4 polyclonal antiserum shows that the NaCl-eluted fraction B and the KI-eluted fraction D contain α4 tubulin, while the unbound fraction A and the NaCl-eluted fraction C do not. Immunoblot results with the antibodies to tyrosinated and nontyrosinated tubulin show that fractions A and B are nontyrosinated whereas fractions C and D are tyrosinated.

Reversed-phase HPLC profile of the endoproteinase Lys-C digest clearly shows that the unbound fraction A contains two sequences which are both specific for α1/2. No other sequence was found to contain either the C-terminal peptide or any other peptide corresponding to α4 tubulin. Immunoblot analysis shows that this fraction reacts only with the monoclonal antibody to nontyrosinated tubulin but not at all with the antibodies to tyrosinated tubulin. Thus, fraction A is essentially the nontyrosinated forms of α1/2 tubulin, that include both the Glu form as well as the Δ2 form. At this time it is not clear about the contribution of the Δ2 form in this fraction; however, the low signal for the Glu^{450} in the sequencing chromatogram may indicate that fraction A may be enriched in the Δ2 form of α1/2. Sequence analysis of fraction B shows that it is a mixture of the nontyrosinated forms of α1/2 and α4. The presence of α4 in this fraction was also confirmed by immunoblotting with antiserum specific for α4 tubulin. It is not known whether this fraction contains any Δ2 tubulin. The analysis of fraction C exhibited two sequences, which are both specific for α1/2. Immunoblotting with antiserum specific for α4 tubulin also confirmed that this fraction is depleted of α4. Thus, fraction C seems to be primarily the tyrosinated form of α1/2 tubulin. Fraction D seems to contain mainly the tyrosinated forms of α4 tubulin. However, it is not clear whether it contains α4 exclusively. Immunoblot results also indicate this. Since this fraction is not functionally active, no further analysis was performed.

It should be mentioned here that the sequencing chromatogram exhibited significantly low signals for some of the amino acid residues in the C-terminal sequences. These residues include the glutamic acid residues Glu^{445}, Glu^{447}, Glu^{449}, and Glu^{450} for α1/2 and Glu^{445}, Glu^{447}, and Glu^{448} for α4. Usually a low signal can be due to the posttranslational modification of a residue; the modified amino acid residue does not show up in the sequencing chromatogram. It is not clear at this point whether the low signal is due to the posttranslational modification of the residue or is just a sequencing error. It is known that Glu^{445} is modified by posttranslational polyglutamylation, while Glu^{450} (for α1/2) and Glu^{448} (for α4) can be deglutamylated to form the corresponding Δ2 form. Thus, a low signal for Glu^{445}, Glu^{449} (in the case of α1/2), and Glu^{445} and Glu^{447} (in the case of α4) can be caused by the covalent modification, while a low signal for Glu^{450} (in the case of α1/2) and Glu^{448} (in the case of α4) can be due to the formation of the corresponding Δ2 form. Extensive mass spectrometric studies are necessary to determine the posttranslational modifications in the tubulin fractions.

It should be mentioned that although the monoclonal antibody AYN.6D10 is specific for the peptide EEGEEY, it exhibits different affinities for different α-tubulin isoforms when used in an immunoaffinity column. The reason is that the specificity of an antibody is determined by Western blotting where the antibody is used at a very high dilution (1/5000 in this case). In contrast, the concentration of antibody in the column is 2−5 mg/mL, which is about 2000-fold higher than the concentration used for Western blotting. Due to this high concentration of the antibody in the immunoaffinity column, a very weak nonspecific affinity for detyrosinated tubulin is observed. Thus, although the epitope for the antibody is the C-terminal tyrosine residue, other factors, such as posttranslational polyglutamylation and/or polyglycylation, that may affect the conformation at the C-terminal region, may also be responsible for the affinity differences. Although it is not clear yet, it seems that the conformation of the COOH-terminal domain for these α-tubulin species may be different, and the affinity of the antibody decreases according to the order: α4-EDEGEEY > α1/2-EEEGEEY > α4-EDEGEE/α1/2-EEEGEE.

The results shown here are quite intriguing in the sense that one can purify the α-tubulin isoforms in the functional state using one immunoaffinity column. The isotypically pure α-tubulin isoforms may be useful for studying microtubule dynamics in vivo. They may also be useful for studying whether they differ in their interactions with motility-related proteins such as dynein and kinesin, or the enzymes responsible for the posttranslational modifications in α-tubulin such as tyrosination, detyrosination, polyglutamylation, and polyglycylation.

The results of the assembly experiment clearly show that unbound fraction A undergoes assembly in the presence of glycerol and Mg^{2+}, as does the unfractionated tubulin. In fact, unbound fraction A assembles slightly better (approximately 10−15%) than unfractionated PC−tubulin. Although this difference in assembly is quite insignificant, it has been found to be reproducible in five different assembly studies using different protein preparations. However, a completely different scenario emerged in the case of NaCl-eluted fractions B and C. Very little assembly is observed in 30 min of incubation.

That fractions B and C are not the denatured fractions of tubulin is confirmed by the fact that they retain the colchicine-binding activity, and also can assemble in the presence of 10 μM taxol to form morphologically normal microtubules (*45*). Furthermore, fraction C can undergo copolymerization with fraction A to form normal microtubules.

The differences in the kinetics of assembly for fractions A, B, and C in the presence of Mg^{2+} may indicate either (a) the isoforms may differ in their interactions with magnesium or (b) different isoforms may differ in their conformational changes upon binding to Mg^{2+} or, in the critical concentration for assembly. Whether the difference in assembly is at the nucleation phase or at the elongation phase remains to be seen.

Although it not known whether the tubulin fractions differ in their polyglutamylation or polyglycylation status, these modifications can certainly affect the assembly property of tubulin. The addition of the glutamyl units as a side chain will certainly increase the net charge at the C-terminal region, while the addition of the glycyl units may lower the net charge to a small extent. Since Mg^{2+} is known to facilitate assembly by counteracting the net negative charge at the

C-terminal region, polyglutamylation in tubulin may result in decreased assembly while polyglycylation may increase it slightly.

In conclusion, I find that the tyrosinated form of α1/2 differs significantly from its nontyrosinated form in the assembly kinetics in the presence of Mg^{2+}. The major part of the nontyrosinated form of α1/2 could assemble by itself (fraction A). However, the tyrosinated form could coassemble along with the detyrosinated form. Such a striking difference in the assembly behavior may indicate that the microtubule assembly in vivo may be regulated by different posttranslational modifications in α-tubulin, which may alter the specific interactions of tubulin with different non-tubulin proteins including microtubule-associated proteins such as MAP1, MAP2, MAP4, and tau. It would be interesting to study whether individual α-tubulin isoforms affect the dynamic behavior of microtubules in vivo. Future studies with these isoforms may shed light on the hitherto unknown roles played by the isoforms and their posttranslational modifications in regulating the microtubule function in vivo.

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